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<p>(21) International Application Number: <b>PCT/US98/08677</b></p> <p>(22) International Filing Date: <b>29 April 1998 (29.04.98)</b></p> <p>(30) Priority Data: 60/045,228 29 April 1997 (29.04.97) US</p> <p>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/045,228 (CIP) Filed on 29 April 1997 (29.04.97)</p> <p>(71) Applicant (<i>for all designated States except US</i>): THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10550 North Torrey Pines Road, La Jolla, CA 92037 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (<i>for US only</i>): JOYCE, Gerald, F. [US/US]; 837 4th Street, Encinitas, CA 92024 (US). BREAKER, Ronald, R. [US/US]; 133 Weatherly Trail, Guilford, CT 06437 (US).</p> <p>(74) Agents: FITTING, Thomas et al.; The Scripps Research Institute, 10550 North Torrey Pines Road, TPC-8, La Jolla, CA 92037 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report.</i></p>	
<p>(54) Title: ENZYMATIC DNA MOLECULES</p> <p>(57) Abstract</p> <p>The present invention discloses deoxyribonucleic acid enzymes — catalytic or enzymatic DNA molecules — capable of cleaving nucleic acid sequences or molecules, particularly RNA, in a site-specific manner, as well as compositions including same. Methods of making and using the disclosed enzymes and compositions are also disclosed.</p>			

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## ENZYMATIC DNA MOLECULES

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DescriptionTECHNICAL FIELD

The present invention relates to nucleic acid enzymes or catalytic (enzymatic) DNA molecules that are capable of 10 cleaving other nucleic acid molecules, particularly RNA. The present invention also relates to compositions containing the disclosed enzymatic DNA molecules and to methods of making and using such enzymes and compositions.

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BACKGROUND

The need for catalysts that operate outside of their native context or which catalyze reactions that are not represented in nature has resulted in the development of "enzyme engineering" technology. The usual route taken in 20 enzyme engineering has been a "rational design" approach, relying upon the understanding of natural enzymes to aid in the construction of new enzymes. Unfortunately, the state of proficiency in the areas of protein structure and chemistry is insufficient to make the generation of novel 25 biological catalysts routine.

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Recently, a different approach for developing novel catalysts has been applied. This method involves the construction of a heterogeneous pool of macromolecules and the application of an *in vitro* selection procedure to isolate molecules from the pool that catalyze the desired reaction. Selecting catalysts from a pool of macromolecules is not dependent on a comprehensive understanding of their structural and chemical properties. Accordingly, this process has been dubbed "irrational design" (Brenner et al., Proc.Natl.Acad.Sci. USA, 89:5381-5383, 1992).

Most efforts to date involving the rational design of enzymatic RNA molecules or ribozymes have not led to molecules with fundamentally new or improved catalytic function. However, the application of irrational design methods via a process we have described as "directed molecular evolution" or "in vitro evolution", which is patterned after Darwinian evolution of organisms in nature, has the potential to lead to the production of DNA molecules that have desirable functional characteristics.

This technique has been applied with varying degrees of success to RNA molecules in solution (see, e.g., Mills et al, Proc.Natl.Acad.Sci. USA, 58:217, 1967; Green et al, Nature, 347:406, 1990; Chowrira et al, Nature, 354:320, 1991; Joyce, Gene, 82:83, 1989; Beaudry et al, Science, 257:635-641, 1992; Robertson et al, Nature, 344:467, 1990), as well as to RNAs bound to a ligand that is attached to a solid support (Tuerk et al, Science, 249:505, 1990; Ellington et al, Nature, 346:818, 1990). It has also been applied to peptides attached directly to a solid support (Lam et al, Nature, 354:82, 1991); and to peptide epitopes expressed within a viral coat protein (Scott et al, Science, 249:386, 1990; Devlin et al, Science, 249:249, 1990; Cwirla et al, Proc.Natl.Acad.Sci. USA, 87:6378, 1990).

It has been more than a decade since the discovery of catalytic RNA (Kruger et al, Cell, 31:147-157, 1982; Guerrier-Takada et al, Cell, 35:849-857, 1983). The list of known naturally-occurring ribozymes continues to grow (see Cech, in The RNA World, Gesteland & Atkins (eds.), pp. 239-269, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1993); Pyle, Science, 261:709-714, 1993; Symons, Curr.Opin.Struct.Biol., 4:322-330, 1994) and, in recent years, has been augmented by synthetic ribozymes obtained through *in vitro* evolution. (See Joyce,

Curr.Opin.Struct.Biol., 4:331-336, 1994; Breaker et al,  
Trends Biotech., 12:268-275, 1994; Chapman et al, Curr.Opin.  
Struct.Biol., 4:618-622, 1994).

It seems reasonable to assume that DNA can have  
5 catalytic activity as well, considering that it contains  
most of the same functional groups as RNA. However, with  
the exception of certain viral genomes and replication  
intermediates, nearly all of the DNA in biological organisms  
occurs as a complete duplex, precluding it from adopting a  
10 complex secondary and tertiary structure. Thus it is not  
surprising that DNA enzymes have not been found in nature.

Until the advent of the present invention, the design,  
synthesis and use of catalytic DNA molecules with  
nucleotide-cleaving capabilities has not been disclosed or  
15 demonstrated. Therefore, the discoveries and inventions  
disclosed herein are particularly significant, in that they  
highlight the potential of *in vitro* evolution as a means of  
designing increasingly more efficient catalytic molecules,  
including enzymatic DNA molecules that cleave other nucleic  
20 acids, particularly RNA.

#### BRIEF SUMMARY OF THE INVENTION

The present invention describes a synthetic or  
25 engineered (i.e., non-naturally-occurring) catalytic DNA  
molecule (or enzymatic DNA molecule) capable of cleaving a  
substrate nucleic acid (NA) sequence at a defined cleavage  
site. The invention also contemplates an enzymatic DNA  
molecule having an endonuclease activity.

A preferred catalytic DNA molecule has site-specific  
30 endonuclease activity specific for a nucleotide sequence  
defining a cleavage site in a preselected substrate nucleic  
acid sequence. The DNA molecule has first and second

substrate binding regions flanking a core region, wherein  
the first substrate binding region has a sequence  
complementary to a first portion of the preselected  
substrate nucleic acid sequence, and the second substrate  
binding region has a sequence complementary to a second  
portion of the preselected substrate nucleic acid sequence.  
The core region has a sequence according to the formula:

(I.) T(stem)'AGC(stem)"Z,

wherein the (stem)' and (stem)" are each three  
sequential nucleotides which when hybridized as a  
(stem)':(stem)" pair comprise three base pairs including at  
least two G:C pairs and wherein Z = WCGR or WCGAA, and W = A  
or T and R = A or G. In a preferred embodiment, formula I  
defines SEQ ID NO 120 (8-17).

Also contemplated is a core region having a sequence  
according to the formula:

(II.) RGGCTAGCXACAAACGA (SEQ ID NO ),

wherein X = T, C or A, and R = A or G. In a preferred  
embodiment, formula I defines SEQ ID NO 121 (10-23).

In another embodiment, the endonuclease activity is  
specific for a nucleotide sequence defining a cleavage site  
comprising single-stranded nucleic acid in a substrate  
nucleic acid sequence. In another preferred variation, the  
cleavage site is double-stranded nucleic acid. Similarly,  
substrate nucleic acid sequences may be single-stranded,  
double-stranded, partially single- or double-stranded,  
looped, or any combination thereof.

In another contemplated embodiment, the substrate  
nucleic acid sequence includes one or more nucleotide  
analogues. In one variation, the substrate nucleic acid  
sequence is a portion of, or attached to, a larger molecule.

In various embodiments, the larger molecule is selected  
from the group consisting of RNA, modified RNA, DNA,

modified DNA, nucleotide analogs, or composites thereof. In another example, the larger molecule comprises a composite of a nucleic acid sequence and a non-nucleic acid sequence.

In another embodiment, the invention contemplates that  
5 a substrate nucleic acid sequence includes one or more nucleotide analogs. A further variation contemplates that the single stranded nucleic acid comprises RNA, DNA, modified RNA, modified DNA, one or more nucleotide analogs, or any composite thereof. In one embodiment of the  
10 disclosed invention, the endonuclease activity comprises hydrolytic cleavage of a phosphoester bond at the cleavage site.

In various preferred embodiments, the catalytic DNA molecules of the present invention are single-stranded in  
15 whole or in part. These catalytic DNA molecules may preferably assume a variety of shapes consistent with their catalytic activity. Thus, in one variation, a catalytic DNA molecule of the present invention includes one or more hairpin loop structures. In yet another variation, a  
20 catalytic DNA molecule may assume a shape similar to that of "hammerhead" ribozymes. In still other embodiments, a catalytic DNA molecule may assume a conformation similar to that of *Tetrahymena thermophila* ribozymes, e.g., those derived from group I introns.

25 Similarly, preferred catalytic DNA molecules of the present invention are able to demonstrate site-specific endonuclease activity irrespective of the original orientation of the substrate molecule. Thus, in one preferred embodiment, an enzymatic DNA molecule of the  
30 present invention is able to cleave a substrate nucleic acid sequence that is separate from the enzymatic DNA molecule -- i.e., it is not linked to the DNAzyme. In another preferred embodiment, an enzymatic DNA molecule is able to cleave an

attached substrate nucleic acid sequence -- i.e., it is able to perform a reaction similar to self-cleavage.

The invention also contemplates enzymatic DNA molecules (catalytic DNA molecules, deoxyribozymes or DNAzymes) having endonuclease activity, whereby the endonuclease activity requires the presence of a divalent cation. In various preferred, alternative embodiments, the divalent cation is selected from the group consisting of  $Pb^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ , and  $Ca^{2+}$ . Another variation contemplates that the endonuclease activity requires the presence of a monovalent cation. In such alternative embodiments, the monovalent cation is preferably selected from the group consisting of  $Na^+$  and  $K^+$ .

In various preferred embodiments of the invention, an enzymatic DNA molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NO 3, SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; SEQ ID NO 20; SEQ ID NO 21; and SEQ ID NO 22. In other preferred embodiments, a catalytic DNA molecule of the present invention comprises a nucleotide sequence selected from the group consisting of SEQ ID NO 23; SEQ ID NO 24; SEQ ID NO 25; SEQ ID NO 26; SEQ ID NO 27; SEQ ID NO 28; SEQ ID NO 29; SEQ ID NO 30; SEQ ID NO 31; SEQ ID NO 32; SEQ ID NO 33; SEQ ID NO 34; SEQ ID NO 35; SEQ ID NO 36; SEQ ID NO 37; SEQ ID NO 38; and SEQ ID NO 39.

Another preferred embodiment contemplates that a catalytic DNA molecule of the present invention comprises a nucleotide sequence selected from the group consisting of SEQ ID NO 50 and SEQ ID NO 51. In yet another preferred embodiment, a catalytic DNA molecule of the present invention comprises a nucleotide sequence selected from the group consisting of SEQ ID NOS 52 through 101. As disclosed herein, catalytic DNA molecules having sequences

substantially similar to those disclosed herein are also contemplated. Thus, a wide variety of substitutions, deletions, insertions, duplications and other mutations may be made to the within-described molecules in order to 5 generate a variety of other useful enzymatic DNA molecules; as long as said molecules display site-specific cleavage activity as disclosed herein, they are within the boundaries of this disclosure.

In a further variation of the present invention, an 10 enzymatic DNA molecule of the present invention preferably has a substrate binding affinity of about 1  $\mu\text{M}$  or less. In another embodiment, an enzymatic DNA molecule of the present invention binds substrate with a  $K_\text{D}$  of less than about 0.1  $\mu\text{M}$ .

15 The present invention also discloses enzymatic DNA molecules having useful turnover rates. In one embodiment, the turnover rate is less than 5  $\text{hr}^{-1}$ ; in a preferred embodiment, the rate is less than about 2  $\text{hr}^{-1}$ ; in a more preferred embodiment, the rate is less than about 1  $\text{hr}^{-1}$ ; in 20 an even more preferred embodiment, the turnover rate is about 0.6  $\text{hr}^{-1}$  or less.

In still another embodiment, an enzymatic DNA molecule 25 of the present invention displays a useful turnover rate wherein the  $k_{\text{obs}}$  is less than 1  $\text{min}^{-1}$ , preferably less than 0.1  $\text{min}^{-1}$ ; more preferably, less than 0.01  $\text{min}^{-1}$ ; and even more preferably, less than 0.005  $\text{min}^{-1}$ . In one variation, the value of  $k_{\text{obs}}$  is approximately 0.002  $\text{min}^{-1}$  or less.

The present invention also contemplates embodiments in which the catalytic rate of the disclosed DNA enzymes is 30 fully optimized. Thus, in various preferred embodiments, the  $K_m$  for reactions enhanced by the presence of  $\text{Mg}^{2+}$  is approximately 0.5-20 mM, preferably about 1-10 mM, and more preferably about 2-5 mM.

The present invention also contemplates an embodiment whereby the nucleotide sequence defining the cleavage site comprises at least one nucleotide. In various other preferred embodiments, a catalytic DNA molecule of the 5 present invention is able to recognize and cleave a nucleotide sequence defining a cleavage site of two or more nucleotides.

In various preferred embodiments, an enzymatic DNA molecule of the present invention comprises a conserved core 10 flanked by one or more substrate binding regions. In one embodiment, an enzymatic DNA molecule includes first and second substrate binding regions. In another embodiment, an enzymatic DNA molecule includes two or more substrate binding regions.

15 As noted previously, preferred catalytic DNA molecules of the present invention may also include a conserved core. In one preferred embodiment, the conserved core comprises one or more conserved regions. In other preferred variations, the one or more conserved regions include a 20 nucleotide sequence selected from the group consisting of CG; CGA; AGCG; AGCCG; CAGCGAT; CTTGTTT; and CTTATT (see, e.g., Figure 3).

In one embodiment of the invention, an enzymatic DNA molecule of the present invention further comprises one or 25 more variable or spacer nucleotides between the conserved regions in the conserved core. In another embodiment, an enzymatic DNA molecule of the present invention further comprises one or more variable or spacer nucleotides between the conserved core and the substrate binding region.

30 In one variation, the first substrate binding region preferably includes a nucleotide sequence selected from the group consisting of CATCTCT; GCTCT; TTGCTTTTT; TGTCTTC; TTGCTGCT; GCCATGCTTT (SEQ ID NO 40); CTCTATTTCT (SEQ ID NO

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41); GTCGGCA; CATCTCTTC; and ACTTCT. In another preferred variation, the second substrate binding region includes a nucleotide sequence selected from the group consisting of TATGTGACGCTA (SEQ ID NO 42); TATAGTCGTA (SEQ ID NO 43);  
5 ATAGCGTATTA (SEQ ID NO 44); ATAGTTACGTCA (SEQ ID NO 45); AATAGTGAAAGTGTT (SEQ ID NO 46); TATAGTGTA; ATAGTCGGT; ATAGGCCCGGT (SEQ ID NO 47); AATAGTGAGGCTTG (SEQ ID NO 48); and ATGNTG.

In various embodiments of the present invention, the  
10 substrate binding regions vary in length. Thus, for example, a substrate binding region may comprise a single nucleotide to dozens of nucleotides. However, it is understood that substrate binding regions of about 3-25 nucleotides in length, preferably about 3-15 nucleotides in  
15 length, and more preferably about 3-10 nucleotides in length are particularly preferred. In various embodiments, the individual nucleotides in the substrate binding regions are able to form complementary base pairs with the nucleotides of the substrate molecules; in other embodiments,  
20 noncomplementary base pairs are formed. A mixture of complementary and noncomplementary base pairing is also contemplated as falling within the scope of the disclosed embodiments of the invention.

In another preferred embodiment, a catalytic DNA  
25 molecule of the present invention may further comprise a third substrate binding region. In some preferred embodiments, the third region includes a nucleotide sequence selected from the group consisting of TGTT; TGTTA; and TGTTAG. Another preferred embodiment of the present  
30 invention discloses an enzymatic DNA molecule further comprising one or more variable or "spacer" regions between the substrate binding regions.

In another disclosed embodiment, the present invention

contemplates a purified, synthetic enzymatic DNA molecule separated from other DNA molecules and oligonucleotides, the enzymatic DNA molecule having an endonuclease activity, wherein the endonuclease activity is specific for a 5 nucleotide sequence defining a cleavage site comprising single- or double-stranded nucleic acid in a substrate nucleic acid sequence. In one variation, a synthetic (or engineered) enzymatic DNA molecule having an endonuclease activity is disclosed, wherein the endonuclease activity is 10 specific for a nucleotide sequence defining a cleavage site consisting essentially of a single- or double-stranded region of a substrate nucleic acid sequence.

In yet another embodiment, the invention contemplates 15 an enzymatic DNA molecule comprising a deoxyribonucleotide polymer having a catalytic activity for hydrolyzing a nucleic acid-containing substrate to produce substrate cleavage products. In one variation, the hydrolysis takes place in a site-specific manner. As noted previously, the polymer may be single-stranded, double-stranded, or some 20 combination of both.

The invention further contemplates that the substrate comprises a nucleic acid sequence. In various embodiments, the nucleic acid sequence substrate comprises RNA, modified RNA, DNA, modified DNA, one or more nucleotide analogs, or 25 composites of any of the foregoing. One embodiment contemplates that the substrate includes a single-stranded segment; still another embodiment contemplates that the substrate is double-stranded.

The present invention also contemplates an enzymatic 30 DNA molecule comprising a deoxyribonucleotide polymer having a catalytic activity for hydrolyzing a nucleic acid-containing substrate to produce a cleavage product. In one variation, the enzymatic DNA molecule has an effective

binding affinity for the substrate and lacks an effective binding affinity for the cleavage product.

In one preferred embodiment, the invention discloses a non-naturally-occurring enzymatic DNA molecule comprising a nucleotide sequence defining a conserved core flanked by 5 recognition domains, variable regions, and spacer regions. Thus, in one preferred embodiment, the nucleotide sequence defines a first variable region contiguous or adjacent to the 5'-terminus of the molecule, a first recognition domain located 3'-terminal to the first variable region, a first spacer region located 3'-terminal to the first recognition domain, a first conserved region located 3'-terminal to the first spacer region, a second spacer region located 3'-terminal to the first conserved region, a second conserved region located 3'-terminal to the second spacer region, a second recognition domain located 3'-terminal to the second conserved region, and a second variable region located 3'-terminal to the second recognition domain.

In another embodiment, the nucleotide sequence preferably defines a first variable region contiguous or adjacent to the 5'-terminus of the molecule, a first recognition domain located 3'-terminal to the first variable region, a first spacer region located 3'-terminal to the first recognition domain, a first conserved region located 3'-terminal to the first spacer region, a second spacer region located 3'-terminal to the first conserved region, a second conserved region located 3'-terminal to the second spacer region, a second recognition domain located 3'-terminal to the second conserved region, a second variable region located 3'-terminal to the second recognition domain, and a third recognition domain located 3'-terminal to the second variable region.

In one variation of the foregoing, the molecule

includes a conserved core region flanked by two substrate binding domains; in another, the conserved core region comprises one or more conserved domains. In other preferred embodiments, the conserved core region further comprises one or more variable or spacer nucleotides. In yet another embodiment, an enzymatic DNA molecule of the present invention further comprises one or more spacer regions.

The present invention further contemplates a wide variety of compositions. For example, compositions including an enzymatic DNA molecule as described hereinabove are disclosed and contemplated herein. In one alternative embodiment, a composition according to the present invention comprises two or more populations of enzymatic DNA molecules as described above, wherein each population of enzymatic DNA molecules is capable of cleaving a different sequence in a substrate. In another variation, a composition comprises two or more populations of enzymatic DNA molecules as described hereinabove, wherein each population of enzymatic DNA molecules is capable of recognizing a different substrate. In various embodiments, it is also preferred that compositions include a monovalent or divalent cation.

The present invention further contemplates methods of generating, selecting, and isolating enzymatic DNA molecules of the present invention. In one variation, a method of selecting enzymatic DNA molecules that cleave a nucleic acid sequence (e.g., RNA) at a specific site comprises the following steps: (a) obtaining a population of putative enzymatic DNA molecules -- whether the sequences are naturally-occurring or synthetic -- and preferably, they are single-stranded DNA molecules; (b) admixing nucleotide-containing substrate sequences with the aforementioned population of DNA molecules to form an admixture; (c) maintaining the admixture for a sufficient period of time

and under predetermined reaction conditions to allow the putative enzymatic DNA molecules in the population to cause cleavage of the substrate sequences, thereby producing substrate cleavage products; (d) separating the population of DNA molecules from the substrate sequences and substrate cleavage products; and (e) isolating DNA molecules that cleave substrate nucleic acid sequences (e.g., RNA) at a specific site from the population.

In a further variation of the foregoing method, the DNA molecules that cleave substrate nucleic acid sequences at a specific site are tagged with an immobilizing agent. In one example, the agent comprises biotin.

In yet another variation of the aforementioned method, one begins by selecting a sequence -- e.g., a predetermined "target" nucleotide sequence -- that one wishes to cleave using an enzymatic DNA molecule engineered for that purpose. Thus, in one embodiment, the pre-selected (or predetermined) "target" sequence is used to generate a population of DNA molecules capable of cleaving substrate nucleic acid sequences at a specific site via attaching or "tagging" it to a deoxyribonucleic acid sequence containing one or more randomized sequences or segments. In one variation, the randomized sequence is about 40 nucleotides in length; in another variation, the randomized sequence is about 50 nucleotides in length. Randomized sequences that are 1-40, 40-50, and 50-100 nucleotides in length are also contemplated by the present invention.

In one embodiment of the present invention, the nucleotide sequence used to generate a population of enzymatic DNA molecules is selected from the group consisting of SEQ ID NO 4, 23, 50 AND 51. In another embodiment, the "target" or "substrate" nucleotide sequence comprises a sequence of one or more ribonucleotides -- see,

e.g., the relevant portions of SEQ ID NOS 4 and 23, and SEQ ID NO 49. It is also contemplated by the present invention that a useful "target" or "substrate" nucleotide sequence may comprise DNA, RNA, or a composite thereof.

5       The invention also contemplates methods as described above, wherein the isolating step further comprises exposing the tagged DNA molecules to a solid surface having avidin linked thereto, whereby the tagged DNA molecules become attached to the solid surface. As before, the substrate may  
10      be RNA, DNA, a composite of both, or a molecule including nucleotide sequences.

15      The present invention also contemplates a method for specifically cleaving a substrate nucleic acid sequence at a particular cleavage site, comprising the steps of (a) providing an enzymatic DNA molecule capable of cleaving a substrate nucleic acid sequence at a specific cleavage site; and (b) contacting the enzymatic DNA molecule with the substrate nucleic acid sequence to cause specific cleavage of the nucleic acid sequence at the cleavage site. In one  
20      variation, the enzymatic DNA molecule is a non-naturally- occurring (or synthetic) DNA molecule. In another variation, the enzymatic DNA molecule is single-stranded.

25      In still another variation of the foregoing method, the substrate comprises a nucleic acid. In various embodiments, the substrate nucleic acid comprises RNA, modified RNA, DNA, modified DNA, one or more nucleotide analogs, or composites of any of the foregoing. In yet another embodiment, the specific cleavage is caused by the endonuclease activity of the enzymatic DNA molecule. Alteration of reaction  
30      conditions -- e.g., the adjustment of pH, temperature, percent cation, percent enzyme, percent substrate, and percent product -- is also contemplated herein.

The present invention also contemplates a method of

cleaving a phosphoester bond, comprising (a) admixing an catalytic DNA molecule capable of cleaving a substrate nucleic acid sequence at a defined cleavage site with a phosphoester bond-containing substrate, to form a reaction  
5 admixture; and (b) maintaining the admixture under predetermined reaction conditions to allow the enzymatic DNA molecule to cleave the phosphoester bond, thereby producing a population of substrate products. In one embodiment, the enzymatic DNA molecule is able to cleave the phosphoester  
10 bond in a site-specific manner. In another embodiment, the method further comprises the steps of (c) separating the products from the catalytic DNA molecule; and (d) adding additional substrate to the enzymatic DNA molecule to form a new reaction admixture.

15 The present invention also contemplates methods of engineering enzymatic DNA molecules that cleave phosphoester bonds. One exemplary method comprises the following steps: (a) obtaining a population of single-stranded DNA molecules; (b) introducing genetic variation into the population to produce a variant population; (c) selecting individuals from the variant population that meet predetermined selection criteria; (d) separating the selected individuals from the remainder of the variant population; and (e) amplifying the  
20 selected individuals.

25

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a selective amplification scheme for isolation of DNAs that cleave a target RNA phosphoester. As shown, double-stranded DNA that contains a stretch of 50  
30 random nucleotides (the molecule with "N<sub>50</sub>" indicated above it) is amplified by PCR, employing a 5'-biotinylated DNA primer that is terminated at the 3' end by an adenosine ribonucleotide (rA). (The biotin label is indicated via the

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encircled letter "B".) This primer is extended by *Taq* polymerase to yield a DNA product that contains a single embedded ribonucleotide. The resulting double-stranded DNA is immobilized on a streptavidin matrix and the  
5 unbiotinylated DNA strand is removed by washing with 0.2 N NaOH. After re-equilibrating the column with a buffered solution, the column is washed with the same solution with added 1 mM PbOAc. DNAs that undergo Pb<sup>2+</sup>-dependent self-cleavage are released from the column, collected in the  
10 eluant, and amplified by PCR. The PCR products are then used to initiate the next round of selective amplification.

Figure 2 illustrates self-cleavage activity of the starting pool of DNA (G0) and populations obtained after the first through fifth rounds of selection (G1 - G5), in the  
15 presence of lead cation (Pb<sup>2+</sup>). The symbol Pre represents 108-nucleotide precursor DNA (SEQ ID NO 4); Clv, 28-nucleotide 5'-cleavage product (SEQ ID NO 5); and M,  
primer 3a (SEQ ID NO 6), which corresponds in length to the 5'-cleavage product.  
20

Figure 3 illustrates the sequence alignment of individual variants isolated from the population after five rounds of selection. The fixed substrate domain is shown at the top, with the target riboadenylate identified via an inverted triangle. Substrate nucleotides that are commonly involved in presumed base-pairing interactions are indicated by vertical bars. Sequences corresponding to the 50 initially-randomized nucleotides are aligned antiparallel to the substrate domain. All of the variants are 3'-terminated by the fixed sequence 5'-CGGTAAGCTTGGCAC-3' (not shown; SEQ  
25 ID NO 1). Nucleotides within the initially-randomized region that are presumed to form base pairs with the substrate domain are indicated on the right and left sides of the Figure; the putative base-pair-forming regions of the  
30

enzymatic DNA molecules are individually boxed in each sequence shown. Conserved regions are illustrated via the two large, centrally-located boxes.

Figures 4A and 4B illustrate DNA-catalyzed cleavage of  
5 an RNA phosphoester in an intermolecular reaction that  
proceeds with catalytic turnover. Figure 4A is a  
diagrammatic representation of the complex formed between  
the 19mer substrate (3'-TCACTATrAGGAAGAGATGG-5', SEQ ID NO  
2) and 38mer DNA enzyme (5'-  
10 ACACATCTCTGAAGTAGCGCCGCCGTATAGTGACGCTA-3', SEQ ID NO 3).  
The substrate contains a single adenosine ribonucleotide  
("rA", adjacent to the arrow), flanked by  
deoxyribonucleotides. The synthetic DNA enzyme is a  
38-nucleotide portion of the most frequently occurring  
15 variant shown in Figure 3. Highly-conserved nucleotides  
located within the putative catalytic domain are "boxed".  
As illustrated, one conserved sequence is "AGCG", while  
another is "CG" (reading in the 5'→3' direction).

Figure 4B shows an Eadie-Hofstee plot used to determine  
20  $K_m$  (negative slope) and  $V_{max}$  (y-intercept) for DNA-catalyzed  
cleavage of [5'-<sup>32</sup>P]-labeled substrate under conditions  
identical to those employed during *in vitro* selection.  
Initial rates of cleavage were determined for reactions  
involving 5 nM DNA enzyme and either 0.125, 0.5, 1, 2, or 4  
25  $\mu\text{M}$  substrate.

Figure 5 is a photographic representation showing a  
polyacrylamide gel demonstrating specific endoribonuclease  
activity of four families of selected catalytic DNAs.  
Selection of a Pb<sup>2+</sup>-dependent family of molecules was  
30 repeated in a side-by-side fashion as a control (first  
group). In the second group, Zn<sup>2+</sup> is used as the cation; in  
group three, the cation is Mn<sup>2+</sup>; and in the fourth group,  
the cation is Mg<sup>2+</sup>. A fifth site on the gel consists of the

cleavage product alone, as a marker.

As noted, there are three lanes within each of the aforementioned four groups. In each group of three lanes, the first lane shows the lack of activity of the selected population in the absence of the metal cation, the second lane shows the observed activity in the presence of the metal cation, and the third lane shows the lack of activity of the starting pool (G0).

Figures 6A and 6B provide two-dimensional illustrations of a "progenitor" catalytic DNA molecule and one of several catalytic DNA molecules obtained via the selective amplification methods disclosed herein, respectively.

Figure 6A illustrates an exemplary molecule from the starting pool, showing the overall configuration of the molecules represented by SEQ ID NO 23. As illustrated, various complementary nucleotides flank the random ( $N_{40}$ ) region. Figure 6B is a diagrammatic representation of one of the  $Mg^{2+}$ -dependent catalytic DNA molecules (or "DNAzymes") generated via the within-described procedures. The location of the ribonucleotide in the substrate nucleic acid is indicated via the arrow in both Figs. 6A and 6B.

Figure 7 illustrates some of the results of ten rounds of *in vitro* selective amplification carried out essentially as described in Example 5 hereinbelow. As shown, two sites and two families of catalysts emerged as displaying the most efficient cleavage of the target sequence. Cleavage conditions were essentially as indicated in Figure 7, namely, 10mM  $Mg^{2+}$ , pH 7.5, and 37°C; data collected after the reaction ran for 2 hours is shown. Cleavage (%) is shown plotted against the number of generations (here, 0 through 10). The number/prevalence of catalytic DNA molecules capable of cleaving the target sequence at the indicated sites in the substrate is illustrated via the

vertical bars, with cleavage at G!UAACUAGAGAU shown by the striped bars, and with cleavage at GUAACUA!GAGAU illustrated via the open (lightly-shaded) bars.

Figure 8 illustrates the nucleotide sequences, cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 and 10-23. Reaction conditions were as shown, namely, 10mM Mg<sup>2+</sup>, pH 7.5, and 37°C. The DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3') -- which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 is shown on the right, with the site of cleavage of the RNA substrate indicated by the arrow. Again, the substrate sequence is indicated. For the 8-17 enzyme, the turnover rate was approximately 0.6 hr<sup>-1</sup>; for the 10-23 enzyme, the turnover rate was approximately 1 hr<sup>-1</sup>. Noncomplementary pairings are indicated with a closed circle (●), whereas complementary pairings are indicated with a vertical line (|).

Figure 9 further illustrates the nucleotide sequences, cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 and 10-23. Reaction conditions were as shown, namely, 10mM Mg<sup>2+</sup>, pH 7.5, and 37°C. As in Figure 8, the DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3') -- which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 is shown on the right, with the site of cleavage of the RNA substrate indicated by

-20-

the arrow. Again, the substrate sequence is indicated. For the 8-17 enzyme,  $k_{obs}$  was approximately  $0.002 \text{ min}^{-1}$ ; for the 10-23 enzyme, the value of  $k_{obs}$  was approximately  $0.01 \text{ min}^{-1}$ . Noncomplementary pairings are indicated with a closed circle (●), whereas complementary pairings are indicated with a vertical line (|).

Figure 10 illustrates a schematic showing the composition of the 8-17 and 10-23 catalytic motifs. The DNA enzyme (bottom strand) binds the RNA substrate (top strand) through complementary Watson-Crick pairing (vertical lines) between unspecified complementary nucleotides (horizontal lines). Cleavage occurs at the position indicated by the arrow, where R = A or G and Y = U or C.

Figure 11 illustrates the catalytic activity of the 10-23 DNA enzyme under multiple-turnover conditions as described in Example 6. Initial velocities were measured over the first 10% of the reaction, employing a fixed concentration of enzyme (0.004 nM) and varying concentrations of substrate (0.02-4 nM). The 17mer RNA substrate, corresponding to the start codon region of HIV-1 gag / pol mRNA, was prepared by in vitro transcription. Reaction conditions: 2 mM MgCl<sub>2</sub>, 150 mM NaCl, pH 7.5, 37 °C. Data from two independent experiments are shown and were fit to the Michaelis-Menten equation:  $v = k_{cat} [E] / (K_m + [S])$ .

Figures 12A and 12B contain two panels that illustrate the effect of variation to the length of the substrate binding regions of a DNA enzyme of the invention, as described in Example 6. The length of the complementary substrate binding region was varied in length (n) from 4 to 13 nucleotides for each the first and second substrate binding region (arm), as noted, and the catalytic activity was measured and expressed as  $k_{cat}$  ( $\text{min}^{-1}$ ) and  $K_m$  (nanomolar [nM]).

Figure 13 illustrates the effect of modifications to the nucleotide residues of a DNA enzyme, as described in Example 6. DNA enzymes were incubated in 10% heat-inactivated fetal bovine serum in RPMI-1640 media at 37°C, comparing unmodified DNA (open circles), inverted thymidylate (filled circles), five 2'-O-Me residues in each arm, (open squares), all 2'-O-Me residues in each arm (filled squares), five P=S residues in core (open triangles), and three P=S residues in each arm (filled triangles).

#### DETAILED DESCRIPTION

##### A. Definitions

As used herein, the term "deoxyribozyme" is used to describe a DNA-containing nucleic acid that is capable of functioning as an enzyme. In the present disclosure, the term "deoxyribozyme" includes endoribonucleases and endodeoxyribonucleases, although deoxyribozymes with endoribonuclease activity are particularly preferred. Other terms used interchangeably with deoxyribozyme herein are "enzymatic DNA molecule", "DNAzyme", or "catalytic DNA molecule", which terms should all be understood to include enzymatically active portions thereof, whether they are produced synthetically or derived from organisms or other sources.

The term "enzymatic DNA molecules" also includes DNA molecules that have complementarity in a substrate-binding region to a specified oligonucleotide target or substrate; such molecules also have an enzymatic activity which is active to specifically cleave the oligonucleotide substrate. Stated in another fashion, the enzymatic DNA molecule is capable of cleaving the oligonucleotide substrate intermolecularly. This complementarity functions to allow

sufficient hybridization of the enzymatic DNA molecule to the substrate oligonucleotide to allow the intermolecular cleavage of the substrate to occur. While one-hundred percent (100%) complementarity is preferred, complementarity in the range of 75-100% is also useful and contemplated by the present invention.

Enzymatic DNA molecules of the present invention may alternatively be described as having nuclease or ribonuclease activity. These terms may be used interchangeably herein.

The term "enzymatic nucleic acid" as used herein encompasses enzymatic RNA or DNA molecules, enzymatic RNA-DNA polymers, and enzymatically active portions or derivatives thereof, although enzymatic DNA molecules are a particularly preferred class of enzymatically active molecules according to the present invention.

The term "endodeoxyribonuclease", as used herein, is an enzyme capable of cleaving a substrate comprised predominantly of DNA. The term "endoribonuclease", as used herein, is an enzyme capable of cleaving a substrate comprised predominantly of RNA.

As used herein, the term "base pair" (bp) is generally used to describe a partnership of adenine (A) with thymine (T) or uracil (U), or of cytosine (C) with guanine (G), although it should be appreciated that less-common analogs of the bases A, T, C, and G (as well as U) may occasionally participate in base pairings. Nucleotides that normally pair up when DNA or RNA adopts a double stranded configuration may also be referred to herein as "complementary bases".

"Complementary nucleotide sequence" generally refers to a sequence of nucleotides in a single-stranded molecule or segment of DNA or RNA that is sufficiently complementary to

that on another single oligonucleotide strand to specifically hybridize to it with consequent hydrogen bonding.

"Nucleotide" generally refers to a monomeric unit of 5 DNA or RNA consisting of a sugar moiety (pentose), a phosphate group, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a "nucleoside". When the nucleoside contains a 10 phosphate group bonded to the 3' or 5' position of the pentose, it is referred to as a nucleotide. A sequence of operatively linked nucleotides is typically referred to herein as a "base sequence" or "nucleotide sequence", and their grammatical equivalents, and is represented herein by 15 a formula whose left to right orientation is in the conventional direction of 5'-terminus to 3'-terminus, unless otherwise specified.

"Nucleotide analog" generally refers to a purine or pyrimidine nucleotide that differs structurally from A, T, 20 G, C, or U, but is sufficiently similar to substitute for the normal nucleotide in a nucleic acid molecule. As used herein, the term "nucleotide analog" encompasses altered bases, different or unusual sugars (i.e. sugars other than the "usual" pentose), or a combination of the two. A 25 listing of exemplary analogs wherein the base has been altered is provided in section C hereinbelow.

"Oligonucleotide or polynucleotide" generally refers to a polymer of single- or double-stranded nucleotides. As used herein, "oligonucleotide" and its grammatical 30 equivalents will include the full range of nucleic acids. An oligonucleotide will typically refer to a nucleic acid molecule comprised of a linear strand of ribonucleotides. The exact size will depend on many factors, which in turn

depends on the ultimate conditions of use, as is well known in the art.

As used herein, the term "physiologic conditions" is meant to suggest reaction conditions emulating those found in mammalian organisms, particularly humans. While variables such as temperature, availability of cations, and pH ranges may vary as described in greater detail below, "physiologic conditions" generally comprise a temperature of about 35-40°C, with 37°C being particularly preferred, as well as a pH of about 7.0-8.0, with 7.5 being particularly preferred, and further comprise the availability of cations, preferably divalent and/or monovalent cations, with a concentration of about 2-15 mM Mg<sup>2+</sup> and 0-1.0 M Na<sup>+</sup> being particularly preferred. "Physiologic conditions", as used herein, may optionally include the presence of free nucleoside cofactor. As noted previously, preferred conditions are described in greater detail below.

#### B. Enzymatic DNA Molecules

In various embodiments, an enzymatic DNA molecule of the present invention may combine one or more modifications or mutations including additions, deletions, and substitutions. In alternative embodiments, such mutations or modifications may be generated using methods which produce random or specific mutations or modifications. These mutations may, for example, change the length of, or alter the nucleotide sequence of, a loop, a spacer region or the recognition sequence (or domain). One or more mutations within one catalytically active enzymatic DNA molecule may be combined with the mutation(s) within a second catalytically active enzymatic DNA molecule to produce a new enzymatic DNA molecule containing the mutations of both molecules.

In other preferred embodiments, an enzymatic DNA molecule of the present invention may have random mutations introduced into it using a variety of methods well known to those skilled in the art. For example, the methods described by Cadwell et al, PCR Methods and Applications, 2:28-33, 1992, are particularly preferred for use as disclosed herein, with some modifications, as described in the Examples that follow. (Also see Cadwell et al, PCR Methods and Applications, 3(Suppl.):S136-S140, 1994.)

According to this modified PCR method, random point mutations may be introduced into cloned genes.

The aforementioned methods have been used, for example, to mutagenize genes encoding ribozymes with a mutation rate of  $0.66\% \pm 0.13\%$  (95% confidence interval) per position, as determined by sequence analysis, with no strong preferences observed with respect to the type of base substitution. This allows the introduction of random mutations at any position in the enzymatic DNA molecules of the present invention.

Another method useful in introducing defined or random mutations is disclosed in Joyce et al, Nucleic Acids Res., 17:711-722, 1989. This latter method involves excision of a template (coding) strand of a double-stranded DNA, reconstruction of the template strand with inclusion of mutagenic oligonucleotides, and subsequent transcription of the partially-mismatched template. This allows the introduction of defined or random mutations at any position in the molecule by including polynucleotides containing known or random nucleotide sequences at selected positions.

Enzymatic DNA molecules of the present invention may be of varying lengths and folding patterns, as appropriate, depending on the type and function of the molecule. For example, enzymatic DNA molecules may be about 15 to about

400 or more nucleotides in length, although a length not exceeding about 250 nucleotides is preferred, to avoid limiting the therapeutic usefulness of molecules by making them too large or unwieldy. In various preferred 5 embodiments, an enzymatic DNA molecule of the present invention is at least about 20 nucleotides in length and, while useful molecules may exceed 100 nucleotides in length, preferred molecules are generally not more than about 100 nucleotides in length.

10 In various therapeutic applications, enzymatic DNA molecules of the present invention comprise the enzymatically active portions of deoxyribozymes. In various embodiments, enzymatic DNA molecules of the present invention preferably comprise not more than about 200 nucleotides. In other embodiments, a deoxyribozyme of the 15 present invention comprises not more than about 100 nucleotides. In still other preferred embodiments, deoxyribozymes of the present invention are about 20-75 nucleotides in length, more preferably about 20-65 20 nucleotides in length. Other preferred enzymatic DNA molecules are about 10-50 nucleotides in length.

In other applications, enzymatic DNA molecules may assume configurations similar to those of "hammerhead" 25 ribozymes. Such enzymatic DNA molecules are preferably no more than about 75-100 nucleotides in length, with a length of about 20-50 nucleotides being particularly preferred.

In general, if one intends to synthesize molecules for use as disclosed herein, the larger the enzymatic nucleic acid molecule is, the more difficult it is to synthesize. 30 Those of skill in the art will certainly appreciate these design constraints. Nevertheless, such larger molecules remain within the scope of the present invention.

It is also to be understood that an enzymatic DNA

molecule of the present invention may comprise enzymatically active portions of a deoxyribozyme or may comprise a deoxyribozyme with one or more mutations, e.g., with one or more base-pair-forming sequences or spacers absent or modified, as long as such deletions, additions or modifications do not adversely impact the molecule's ability to perform as an enzyme.

The recognition domain of an enzymatic DNA molecule of the present invention typically comprises two nucleotide sequences flanking a catalytic domain, and typically contains a sequence of at least about 3 to about 30 bases, preferably about 6 to about 15 bases, which are capable of hybridizing to a complementary sequence of bases within the substrate nucleic acid giving the enzymatic DNA molecule its high sequence specificity. Modification or mutation of the recognition site via well-known methods allows one to alter the sequence specificity of an enzymatic nucleic acid molecule. (See Joyce et al, Nucleic Acids Res., 17:711-712, 1989.)

Enzymatic nucleic acid molecules of the present invention also include those with altered recognition sites or domains. In various embodiments, these altered recognition domains confer unique sequence specificities on the enzymatic nucleic acid molecule including such recognition domains. The exact bases present in the recognition domain determine the base sequence at which cleavage will take place. Cleavage of the substrate nucleic acid occurs within the recognition domain. This cleavage leaves a 2', 3', or 2',3'-cyclic phosphate group on the substrate cleavage sequence and a 5' hydroxyl on the nucleotide that was originally immediately 3' of the substrate cleavage sequence in the original substrate. Cleavage can be redirected to a site of choice by changing

the bases present in the recognition sequence (internal guide sequence). See Murphy et al, Proc. Natl. Acad. Sci. USA, 86:9218-9222, 1989.

Moreover, it may be useful to add a polyamine to facilitate recognition and binding between the enzymatic DNA molecule and its substrate. Examples of useful polyamines include spermidine, putrescine or spermine. A spermidine concentration of about 1 mM may be effective in particular embodiments, while concentrations ranging from about 0.1 mM to about 10 mM may also be useful.

In various alternative embodiments, an enzymatic DNA molecule of the present invention has an enhanced or optimized ability to cleave nucleic acid substrates, preferably RNA substrates. As those of skill in the art will appreciate, the rate of an enzyme-catalyzed reaction varies depending upon the substrate and enzyme concentrations and, in general, levels off at high substrate or enzyme concentrations. Taking such effects into account, the kinetics of an enzyme-catalyzed reaction may be described in the following terms, which define the reaction.

The enhanced or optimized ability of an enzymatic DNA molecule of the present invention to cleave an RNA substrate may be determined in a cleavage reaction with varying amounts of labeled RNA substrate in the presence of enzymatic DNA molecule. The ability to cleave the substrate is generally defined by the catalytic rate ( $k_{cat}$ ) divided by the Michaelis constant ( $K_m$ ). The symbol  $k_{cat}$  represents the maximal velocity of an enzyme reaction when the substrate approaches a saturation value.  $K_m$  represents the substrate concentration at which the reaction rate is one-half maximal.

For example, values for  $K_m$  and  $k_{cat}$  may be determined in this invention by experiments in which the substrate

concentration [S] is in excess over enzymatic DNA molecule concentration [E]. Initial rates of reaction ( $v_0$ ) over a range of substrate concentrations are estimated from the initial linear phase, generally the first 5% or less of the reaction. Data points are fit by a least squares method to a theoretical line given by the equation:  $v = -K_M(v_0/[S]) + V_{max}$ . Thus,  $k_{cat}$  and  $K_M$  are determined by the initial rate of reaction,  $v_0$ , and the substrate concentration [S].

In various alternative embodiments, an enzymatic DNA molecule of the present invention has an enhanced or optimized ability to cleave nucleic acid substrates, preferably RNA substrates. In preferred embodiments, the enhanced or optimized ability of an enzymatic DNA molecule to cleave RNA substrates shows about a 10- to 10<sup>9</sup>-fold improvement over the uncatalyzed rate. In more preferred embodiments, an enzymatic DNA molecule of the present invention is able to cleave RNA substrates at a rate that is about 10<sup>3</sup>- to 10<sup>7</sup>-fold improved over "progenitor" species. In even more preferred embodiments, the enhanced or optimized ability to cleave RNA substrates is expressed as a 10<sup>4</sup>- to 10<sup>6</sup>-fold improvement over the progenitor species. One skilled in the art will appreciate that the enhanced or optimized ability of an enzymatic DNA molecule to cleave nucleic acid substrates may vary depending upon the selection constraints applied during the *in vitro* evolution procedure of the invention.

Various preferred methods of modifying deoxyribozymes and other enzymatic DNA molecules and nucleases of the present invention are further described in Examples 1-3 hereinbelow.

C. Nucleotide Analogs

As noted above, the term "nucleotide analog" as

used herein generally refers to a purine or pyrimidine nucleotide that differs structurally from A, T, G, C, or U, but is sufficiently similar to substitute for such "normal" nucleotides in a nucleic acid molecule. As used herein, the 5 term "nucleotide analog" encompasses altered bases, different (or unusual) sugars, altered phosphate backbones, or any combination of these alterations. Examples of nucleotide analogs useful according to the present invention include those listed in the following Table, most of which 10 are found in the approved listing of modified bases at 37 CFR §1.822 (which is incorporated herein by reference).

**Table 1**  
**Nucleotide Analogs**

15

	<u>Abbreviation</u>	<u>Description</u>
	ac4c	4-acetylcytidine
	chm5u	5-(carboxyhydroxymethyl)uridine
20	cm	2'-O-methylcytidine
	cmmnm5s2u	5-carboxymethylaminomethyl-2-thiouridine
	d	dihydrouridine
	fm	2'-O-methylpseudouridine
	galq	B, D-galactosylqueosine
25	gm	2'-O-methylguanosine
	I	inosine
	i6a	N6-isopentenyladenosine
	m1a	1-methyladenosine
	m1f	1-methylpseudouridine
30	m1g	1-methylguanosine
	m1l	1-methylinosine
	m22g	2,2-dimethylguanosine
	m2a	2-methyladenosine

	m2g	2-methylguanosine
	m3c	3-methylcytidine
	m5c	5-methylcytidine
	m6a	N6-methyladenosine
5	m7g	7-methylguanosine
	mam5u	5-methylaminomethyluridine
	mam5s2u	5-methoxyaminomethyl-2-thiouridine
	manq	$\beta$ , D-mannosylmethyluridine
	mcm5s2u	5-methoxycarbonylmethyluridine
10	mo5u	5-methoxyuridine
	ms2i6a	2-methylthio-N6-isopentenyladenosine
	ms2t6a	N-((9- $\beta$ -D-ribofuranosyl-2-methyl thiopurine-6-yl)carbamoyl)threonine
	mt6a	N-((9- $\beta$ -D-ribofuranosylpurine-6-yl)N- methyl-carbamoyl)threonine
15	mv	uridine-5-oxyacetic acid methylester
	o5u	uridine-5-oxyacetic acid (v)
	osyw	wybutoxosine
	p	pseudouridine
20	q	queosine
	s2c	2-thiocytidine
	s2t	5-methyl-2-thiouridine
	s2u	2-thiouridine
	s4u	4-thiouridine
25	t	5-methyluridine
	t6a	N-((9- $\beta$ -D-ribofuranosylpurine-6- yl)carbamoyl)threonine
	tm	2'-O-methyl-5-methyluridine
	um	2'-O-methyluridine
30	yw	wybutoxosine
	x	3-(3-amino-3-carboxypropyl)uridine, (acp3)u
	araU	$\beta$ , D-arabinosyl

araT

 $\beta$ , D-arabinosyl

5        Other useful analogs include those described in published international application no. WO 92/20823 (the disclosures of which are incorporated herein by reference), or analogs made according to the methods disclosed therein. Analogs described in DeMesmaeker et al, Angew. Chem. Int.  
10      Ed. Engl., 33:226-229, 1994; DeMesmaeker et al, Synlett 733-736 (Oct. 1993); Nielsen et al, Science, 254:1497-1500, 1991; and Idziak et al, Tetrahedron Letters, 34:5417-5420, 1993, are also useful according to the within-disclosed invention and said disclosures are incorporated by reference  
15      herein.

D. Methods of Engineering Enzymatic DNA Molecules

20      The present invention also contemplates methods of producing nucleic acid molecules having a predetermined activity. In one preferred embodiment, the nucleic acid molecule is an enzymatic DNA molecule. In another variation, the desired activity is a catalytic activity.

25      In one embodiment, the present invention contemplates methods of synthesizing enzymatic DNA molecules that may then be "engineered" to catalyze a specific or predetermined reaction. Methods of preparing enzymatic DNA molecules are described herein; see, e.g., Examples 1-3 hereinbelow. In other embodiments, an enzymatic DNA molecule of the present invention may be engineered to bind small molecules or  
30      ligands, such as adenosine triphosphate (ATP). (See Sassanfar et al, Nature, 364:550-553, 1993.)

35      In another embodiment, the present invention contemplates that a population of enzymatic DNA molecules may be subjected to mutagenizing conditions to produce a diverse population of mutant enzymatic DNA molecules (which

may alternatively be called "deoxyribozymes" or "DNAzymes"). Thereafter, enzymatic DNA molecules having desired characteristics are selected and/or separated from the population and are subsequently amplified.

5        Alternatively, mutations may be introduced in the enzymatic DNA molecule by altering the length of the recognition domains of the enzymatic DNA molecule. The recognition domains of the enzymatic DNA molecule associate with a complementary sequence of bases within a substrate  
10      nucleic acid sequence. Methods of altering the length of the recognition domains are known in the art and include PCR, for example; useful techniques are described further in the Examples below.

15      Alteration of the length of the recognition domains of an enzymatic DNA molecule may have a desirable effect on the binding specificity of the enzymatic DNA molecule. For example, an increase in the length of the recognition domains may increase binding specificity between the enzymatic DNA molecule and the complementary base sequences  
20      of an oligonucleotide in a substrate, or may enhance recognition of a particular sequence in a hybrid substrate. In addition, an increase in the length of the recognition domains may also increase the affinity with which it binds to substrate. In various embodiments, these altered  
25      recognition domains in the enzymatic DNA molecule confer increased binding specificity and affinity between the enzymatic DNA molecule and its substrate.

It has recently been noted that certain  
30      oligonucleotides are able to recognize and bind molecules other than oligonucleotides with complementary sequences. These oligonucleotides are often given the name "aptamers". For example, Ellington et al describe RNA molecules that are able to bind a variety of organic dyes (Nature, 346:818-822,

1990), while Bock et al describe ssDNA molecules that bind  
human thrombin (Nature, 355:564-566, 1992). Similarly,  
Jellinek et al describe RNA ligands to basic fibroblast  
growth factor (Proc.Natl.Acad.Sci. USA, 90:11227-11231,  
5 1993). Thus, it is further contemplated herein that the  
catalytically active DNA enzymes of the present invention  
may be engineered according to the within-described methods  
to display a variety of capabilities typically associated  
with aptamers.

10 One of skill in the art should thus appreciate that the  
enzymatic DNA molecules of this invention can be altered at  
any nucleotide sequence, such as the recognition domains, by  
various methods disclosed herein, including PCR and 3SR  
(self-sustained sequence replication -- see Example 1  
15 below). For example, additional nucleotides can be added to  
the 5' end of the enzymatic DNA molecule by including  
additional nucleotides in the primers.

20 Enzymatic DNA molecules of the present invention may  
also be prepared or engineered in a more non-random fashion  
via use of methods such as site-directed mutagenesis. For  
example, site-directed mutagenesis may be carried out  
essentially as described in Morinaga et al, Biotechnology,  
2:636, 1984, modified as described herein, for application  
to deoxyribozymes. Useful methods of engineering enzymatic  
25 DNA molecules are further described in the Examples below.

30 In one disclosed embodiment, an enzymatic DNA molecule  
of the present invention comprises a conserved core flanked  
by two substrate binding (or recognition) domains or  
sequences that interact with the substrate through  
base-pairing interactions. In various embodiments, the  
conserved core comprises one or more conserved domains or  
sequences. In another variation, an enzymatic DNA molecule  
further comprises a "spacer" region (or sequence) between

the regions (or sequences) involved in base pairing. In still another variation, the conserved core is "interrupted" at various intervals by one or more less-conserved variable or "spacer" nucleotides.

5       In various embodiments, the population of enzymatic DNA molecules is made up of at least 2 different types of deoxyribozyme molecules. For example, in one variation, the molecules have differing sequences. In another variation, the deoxyribozymes are nucleic acid molecules having a  
10      nucleic acid sequence defining a recognition domain that is contiguous or adjacent to the 5'-terminus of the nucleotide sequence. In various alternative embodiments, enzymatic DNA molecules of the present invention may further comprise one or more spacer regions located 3'-terminal to the  
15      recognition domains, one or more loops located 3'-terminal to the recognition domains and/or spacer regions. In other variations, a deoxyribozyme of the present invention may comprise one or more regions which are capable of hybridizing to other regions of the same molecule. Other  
20      characteristics of enzymatic DNA molecules produced according to the presently-disclosed methods are described elsewhere herein.

In other embodiments, mutagenizing conditions include conditions that introduce either defined or random  
25      nucleotide substitutions within an enzymatic DNA molecule. Examples of typical mutagenizing conditions include conditions disclosed in other parts of this specification and the methods described by Joyce et al, Nucl. Acids Res., 17:711-722, 1989; Joyce, Gene, 82:83-87, 1989; and Beaudry  
30      et al, Science, 257:635-41, 1992.

In still other embodiments, a diverse population of mutant enzymatic nucleic acid molecules of the present invention is one that contains at least 2 nucleic acid

molecules that do not have the exact same nucleotide sequence. In other variations, from such a diverse population, an enzymatic DNA molecule or other enzymatic nucleic acid having a predetermined activity is then  
5 selected on the basis of its ability to perform the predetermined activity. In various embodiments, the predetermined activity comprises, without limitation, enhanced catalytic activity, decreased  $K_m$ , enhanced substrate binding ability, altered substrate specificity,  
10 and the like.

Other parameters which may be considered aspects of enzyme performance include catalytic activity or capacity, substrate binding ability, enzyme turnover rate, enzyme sensitivity to feedback mechanisms, and the like. In  
15 certain aspects, substrate specificity may be considered an aspect of enzyme performance, particularly in situations in which an enzyme is able to recognize and bind two or more competing substrates, each of which affects the enzyme's performance with respect to the other substrate(s).

Substrate specificity, as used herein, may refer to the specificity of an enzymatic nucleic acid molecule as described herein for a particular substrate, such as one comprising ribonucleotides only, deoxyribonucleotides only, or a composite of both. Substrate molecules may also  
25 contain nucleotide analogs. In various embodiments, an enzymatic nucleic acid molecule of the present invention may preferentially bind to a particular region of a hybrid or non-hybrid substrate.

The term or parameter identified herein as "substrate specificity" may also include sequence specificity; i.e., an enzymatic nucleic acid molecule of the present invention may "recognize" and bind to a nucleic acid substrate having a particular nucleic acid sequence. For example, if the  
30

substrate recognition domains of an enzymatic nucleic acid molecule of the present invention will only bind to substrate molecules having a series of one or two ribonucleotides (e.g., rA) in a row, then the enzymatic 5 nucleic acid molecule will tend not to recognize or bind nucleic acid substrate molecules lacking such a sequence.

With regard to the selection process, in various embodiments, selecting includes any means of physically separating the mutant enzymatic nucleic acids having a 10 predetermined activity from the diverse population of mutant enzymatic nucleic acids. Often, selecting comprises separation by size, by the presence of a catalytic activity, or by hybridizing the mutant nucleic acid to another nucleic acid, to a peptide, or some other molecule that is either in 15 solution or attached to a solid matrix.

In various embodiments, the predetermined activity is such that the mutant enzymatic nucleic acid having the predetermined activity becomes labeled in some fashion by virtue of the activity. For example, the predetermined 20 activity may be an enzymatic DNA molecule activity whereby the activity of the mutant enzymatic nucleic acid upon its substrate causes the mutant enzymatic nucleic acid to become covalently linked to it. The mutant enzymatic nucleic acid is then selected by virtue of the covalent linkage.

25 In other embodiments, selecting a mutant enzymatic nucleic acid having a predetermined activity includes amplification of the mutant enzymatic nucleic acid (see Joyce, Gene, 82:83-87, 1989; Beaudry et al, Science, 257:635-41, 1992). Other methods of selecting an enzymatic 30 nucleic acid molecule having a predetermined characteristic or activity are described in the Examples section.

E. Compositions

- The invention also contemplates compositions containing one or more types or populations of enzymatic DNA molecules of the present invention; e.g., different types or populations may recognize and cleave different nucleotide sequences. Compositions may further include a ribonucleic acid-containing substrate. Compositions according to the present invention may further comprise lead ion, magnesium ion, or other divalent or monovalent cations, as discussed herein.
- 10 Preferably, the enzymatic DNA molecule is present at a concentration of about 0.05  $\mu$ M to about 2  $\mu$ M. Typically, the enzymatic DNA molecule is present at a concentration ratio of enzymatic DNA molecule to substrate of from about 1:5 to about 1:50. More preferably, the enzymatic DNA
- 15 molecule is present in the composition at a concentration of about 0.1  $\mu$ M to about 1  $\mu$ M. Even more preferably, compositions contain the enzymatic DNA molecule at a concentration of about 0.1  $\mu$ M to about 0.5  $\mu$ M. Preferably, the substrate is present in the composition at a
- 20 concentration of about 0.5  $\mu$ M to about 1000  $\mu$ M.

One skilled in the art will understand that there are many sources of nucleic acid-containing substrates including naturally-occurring and synthetic sources. Sources of suitable substrates include, without limitation, a variety of viral and retroviral agents, including HIV-1, HIV-2, HTLV-I, and HTLV-II.

Other suitable substrates include, without limitation, viral and retroviral agents including those comprising or produced by picornaviruses, hepadnaviridae (e.g., HBV, HCV), papillomaviruses (e.g., HPV), gammaherpesvirinae (e.g., EBV), lymphocryptoviruses, leukemia viruses (e.g., HTLV-I and -II), flaviviruses, togaviruses, herpesviruses (including alphaherpesviruses and betaherpesviruses),

cytomegaloviruses (CMV), influenza viruses, and viruses and retroviruses contributing to immunodeficiency diseases and syndromes (e.g., HIV-1 and -2). In addition, suitable substrates include viral and retroviral agents which infect 5 non-human primates and other animals including, without limitation, the simian and feline immunodeficiency viruses and bovine leukemia viruses.

Magnesium ion, lead ion, or another suitable monovalent or divalent cation, as described previously, may also be 10 present in the composition, at a concentration ranging from about 1-100 mM. More preferably, the preselected ion is present in the composition at a concentration of about 2 mM to about 50 mM, with a concentration of about 5 mM being particularly preferred. One skilled in the art will 15 understand that the ion concentration is only constrained by the limits of solubility of its source (e.g. magnesium) in aqueous solution and a desire to have the enzymatic DNA molecule present in the same composition in an active conformation.

20 The invention also contemplates compositions containing an enzymatic DNA molecule of the present invention, hybrid deoxyribonucleotide-ribonucleotide molecules, and magnesium or lead ion in concentrations as described hereinabove. As noted previously, other monovalent or divalent ions (e.g., 25  $\text{Ca}^{2+}$ ) may be used in place of magnesium.

Also contemplated by the present invention are 30 compositions containing an enzymatic DNA molecule of the present invention, nucleic acid-containing substrate (e.g. RNA), and a preselected ion at a concentration of greater than about 1 millimolar, wherein said substrate is greater in length than the recognition domains present on the enzymatic DNA molecule.

In one variation, a composition comprises an enzymatic

DNA molecule-substrate complex, wherein base pairing between an enzymatic DNA molecule and its substrate is contiguous. In another embodiment, base pairing between an enzymatic DNA molecule and its substrate is interrupted by one or more noncomplementary pairs. In a variety of alternative embodiments, a composition of the present invention may further comprise a monovalent cation, a divalent cation, or both.

In another variation, an enzymatic DNA molecule of the present invention is capable of functioning efficiently in the presence or absence of a divalent cation. In one variation, a divalent cation is present and comprises  $Pb^{2+}$ ,  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ , or  $Ca^{2+}$ . Alternatively, an enzymatic DNA molecule of the present invention is capable of functioning efficiently in the presence or absence of monovalent cations. It is anticipated that monovalent or divalent cation concentrations similar to those described herein for  $Pb^{2+}$  or  $Mg^{2+}$  will be useful as disclosed herein.

Optionally, monovalent cations may also be present in addition to, or as "alternatives" for, divalent cations. For example, monovalent cations such as sodium ( $Na^+$ ) or potassium ( $K^+$ ) may be present, either as dissociated ions or in the form of dissociable compounds such as NaCl or KCl.

In one embodiment, the concentration of monovalent cation present in the composition ranges from 0 - 1.0 M. In another embodiment, a monovalent cation is present in a concentration ranging from about 0-200 mM. In other embodiments, monovalent cations are present in a concentration ranging from about 1-100 mM. Alternatively, the concentration of monovalent cations ranges from about 2 mM - 50 mM. In still other embodiments, the concentration ranges from about 2 mM - 25 mM.

F. Methods of Using Enzymatic DNA Molecules

Methods for using enzymatic DNA molecules as disclosed herein cover many different utilities as are well known in the art for prior art enzymatic and/or antisense oligonucleotides. As discussed previously, molecules capable of cleaving the bonds linking neighboring nucleic acids (e.g., phosphoester bonds) have numerous uses encompassing a wide variety of applications. For example, enzymatic DNA molecules having the within-disclosed capabilities, structures, and/or functions are useful in pharmaceutical and medical products (e.g., for wound debridement, clot dissolution, etc.), as well as in household items (e.g., detergents, dental hygiene products, meat tenderizers). For inactivating target nucleic acid sequences, such as mRNA in vitro and in vivo. Industrial utility of the within-disclosed compounds, compositions and methods is also contemplated and well within the scope of the present invention.

The present invention also describes useful methods for cleaving any single-stranded, looped, partially or fully double-stranded nucleic acid; the majority of these methods employ the novel enzymatically active nucleic acid molecules of the present invention. In various embodiments, the single-stranded nucleic acid segment or portion of the substrate (or the entire substrate itself) comprises DNA, modified DNA, RNA, modified RNA, or composites thereof. Preferably, the nucleic acid substrate need only be single-stranded at or near the substrate cleavage sequence so that an enzymatic nucleic acid molecule of the present invention can hybridize to the substrate cleavage sequence by virtue of the enzyme's recognition sequence.

A nucleic acid substrate that can be cleaved by a method of this invention may be chemically synthesized or

enzymatically produced, or it may be isolated from various sources such as phages, viruses, prokaryotic cells, or eukaryotic cells, including animal cells, plant cells, yeast cells and bacterial cells. Chemically synthesized single-  
5 and double-stranded nucleic acids are commercially available from many sources including, without limitation, Research Genetics (Huntsville, AL).

RNA substrates may also be synthesized using an Applied Biosystems (Foster City, CA) oligonucleotide synthesizer  
10 according to the manufacturer's instructions. Single-stranded phage are also a source of nucleic acid substrates.  
(See Messing et al, Proc.Natl.Acad.Sci. USA, 74:3642-3646,  
1977, and Yanisch-Perron et al, Gene, 33:103-119, 1985).  
Bacterial cells containing single-stranded phage would also  
15 be a ready source of suitable single-stranded nucleic acid substrates.

Single-stranded RNA cleavable by a method of the present invention could be provided by any of the RNA viruses such as the picornaviruses, togaviruses,  
20 orthomyxoviruses, paramyxoviruses, rhabdoviruses, coronaviruses, arenaviruses or retroviruses. As noted previously, a wide variety of prokaryotic and eukaryotic cells may also be excellent sources of suitable nucleic acid substrates.

The methods of this invention may be used on single-stranded nucleic acids or single-stranded portions of looped or double-stranded nucleic acids that are present inside a cell, including eukaryotic, prokaryotic, plant, animal, yeast or bacterial cells. Under these conditions an  
25 enzymatic nucleic acid molecule (e.g., an enzymatic DNA molecule or deoxyribozyme) of the present invention could act as an anti-viral agent or a regulator of gene expression. Examples of such uses of enzymatic DNA

molecules of the present invention are described further hereinbelow.

In the majority of methods of the present invention, cleavage of single-stranded nucleic acids occurs at the 3'-terminus of a predetermined base sequence. This predetermined base sequence or substrate cleavage sequence typically contains from 1 to about 10 nucleotides. In other preferred embodiments, an enzymatic DNA molecule of the present invention is able to recognize nucleotides either upstream, or upstream and downstream of the cleavage site. In various embodiments, an enzymatic DNA molecule is able to recognize about 2-10 nucleotides upstream of the cleavage site; in other embodiments, an enzymatic DNA molecule is able to recognize about 2-10 nucleotides upstream and about 2-10 nucleotides downstream of the cleavage site. Other preferred embodiments contemplate an enzymatic DNA molecule that is capable of recognizing a nucleotide sequence up to about 30 nucleotides in length, with a length up to about 20 nucleotides being even more preferred.

The within-disclosed methods allow cleavage at any nucleotide sequence by altering the nucleotide sequence of the recognition domains of the enzymatic DNA molecule. This allows cleavage of single-stranded nucleic acid in the absence of a restriction endonuclease site at the selected position.

An enzymatic DNA molecule of the present invention may be separated from any portion of the single-stranded nucleic acid substrate that remains attached to the enzymatic DNA molecule by site-specific hydrolysis at the appropriate cleavage site. Separation of the enzymatic DNA molecule from the substrate (or "cleavage product") allows the enzymatic DNA molecule to carry out another cleavage reaction.

Generally, the nucleic acid substrate is treated under appropriate nucleic acid cleaving conditions -- preferably, physiologic conditions -- with an effective amount of an enzymatic DNA molecule of the present invention. If the 5 nucleic acid substrate comprises DNA, cleaving conditions may include the presence of a divalent cation at a concentration of about 2-10 mM.

An effective amount of an enzymatic DNA molecule is the amount required to cleave a predetermined base sequence 10 present within the single-stranded nucleic acid.

Preferably, the enzymatic DNA molecule is present at a molar ratio of DNA molecule to substrate cleavage sites of 1 to 20. This ratio may vary depending on the length of treating and efficiency of the particular enzymatic DNA molecule 15 under the particular nucleic acid cleavage conditions employed.

Thus, in one preferred embodiment, treating typically involves admixing, in aqueous solution, the RNA-containing substrate and the enzyme to form a cleavage admixture, and 20 then maintaining the admixture thus formed under RNA cleaving conditions for a time period sufficient for the enzymatic DNA molecule to cleave the RNA substrate at any of the predetermined nucleotide sequences present in the RNA. In various embodiments, a source of ions is also provided -- 25 i.e. monovalent or divalent cations, or both.

In one embodiment of the present invention, the amount of time necessary for the enzymatic DNA molecule to cleave the single-stranded nucleic acid has been predetermined. The amount of time is from about 1 minute to about 24 hours 30 and will vary depending upon the concentration of the reactants and the temperature of the reaction. Usually, this time period is from about 10 minutes to about 2 hours such that the enzymatic DNA molecule cleaves the single-

stranded nucleic acid at any of the predetermined nucleotide sequences present.

The invention further contemplates that the nucleic acid cleaving conditions include the presence of a source of 5 divalent cations (e.g., PbOAc) at a concentration of about 2-100 mM. Typically, the nucleic acid cleaving conditions include divalent cation at a concentration of about 2 mM to about 10 mM, with a concentration of about 5 mM being particularly preferred.

10 The optimal cationic concentration to include in the nucleic acid cleaving conditions can be easily determined by determining the amount of single-stranded nucleic acid cleaved at a given cation concentration. One skilled in the art will understand that the optimal concentration may vary depending on the particular enzymatic DNA molecule employed.

15 The present invention further contemplates that the nucleic acid cleaving conditions include a pH of about pH 6.0 to about pH 9.0. In one preferred embodiment, the pH ranges from about pH 6.5 to pH 8.0. In another preferred embodiment, the pH emulates physiological conditions, i.e., the pH is about 7.0-7.8, with a pH of about 7.5 being particularly preferred.

20 One skilled in the art will appreciate that the methods of the present invention will work over a wide pH range so long as the pH used for nucleic acid cleaving is such that the enzymatic DNA molecule is able to remain in an active conformation. An enzymatic DNA molecule in an active conformation is easily detected by its ability to cleave 25 single-stranded nucleic acid at a predetermined nucleotide sequence.

30 In various embodiments, the nucleic acid cleaving conditions also include a variety of temperature ranges. As noted previously, temperature ranges consistent with

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physiological conditions are especially preferred, although temperature ranges consistent with industrial applications are also contemplated herein. In one embodiment, the temperature ranges from about 15°C to about 60°C. In 5 another variation, the nucleic acid cleaving conditions include a temperature ranging from about 30°C to about 56°C. In yet another variation, nucleic acid cleavage conditions include a temperature from about 35°C to about 50°C. In a preferred embodiment, nucleic acid cleavage conditions 10 comprise a temperature range of about 37°C to about 42°C. The temperature ranges consistent with nucleic acid cleaving conditions are constrained only by the desired cleavage rate and the stability of that particular enzymatic DNA molecule at that particular temperature.

15 In various methods, the present invention contemplates nucleic acid cleaving conditions including the presence of a polyamine. Polyamines useful for practicing the present invention include spermidine, putrescine, spermine and the like. In one variation, the polyamine is present at a 20 concentration of about .01 mM to about 10 mM. In another variation, the polyamine is present at a concentration of about 1 mM to about 10 mM. Nucleic acid cleavage conditions may also include the presence of polyamine at a 25 concentration of about 2 mM to about 5 mM. In various preferred embodiments, the polyamine is spermidine.

#### G. Vectors

The present invention also features expression vectors including a nucleic acid segment encoding an 30 enzymatic DNA molecule of the present invention situated within the vector, preferably in a manner which allows expression of that enzymatic DNA molecule within a target cell (e.g., a plant or animal cell).

Thus, in general, a vector according to the present invention preferably includes a plasmid, cosmid, phagemid, virus, or phage vector. Preferably, suitable vectors comprise single-stranded DNA (ssDNA) -- e.g., circular  
5 phagemid ssDNA. It should also be appreciated that useful vectors according to the present invention need not be circular.

In one variation, nucleotide sequences flanking each of the additional enzymatic DNA molecule-encoding sequences are  
10 preferably provided, which sequences may be recognized by the first enzymatic DNA molecule. The intervening or flanking sequences preferably comprise at least 1 nucleotide; more preferably, intervening or flanking sequences are about 2-20 nucleotides in length, with  
15 sequences of about 5-10 nucleotides in length being particularly preferred.

The addition of polynucleotide tails may also be useful to protect the 3' end of an enzymatic DNA molecule according to the present invention. These may be provided by  
20 attaching a polymeric sequence by employing the enzyme terminal transferase.

A vector according to the present invention includes two or more enzymatic DNA molecules. In one embodiment, a first enzymatic DNA molecule has intramolecular cleaving  
25 activity and is able to recognize and cleave nucleotide sequences to release other enzymatic DNA sequences; i.e., it is able to function to "release" other enzymatic DNA molecules from the vector. For example, a vector is  
30 preferably constructed so that when the first enzymatic DNA molecule is expressed, that first molecule is able to cleave nucleotide sequences flanking additional nucleotide sequences encoding a second enzymatic DNA molecule, a third enzymatic DNA molecule, and so forth. Presuming said first

enzymatic DNA molecule (i.e., the "releasing" molecule) is able to cleave oligonucleotide sequences intramolecularly, the additional (e.g. second, third, and so on) enzymatic DNA molecules (i.e., the "released" molecules) need not possess characteristics identical to the "releasing" molecule. For example, in one embodiment, the "released" (i.e., the second, third, etc.) enzymatic DNA molecules are able to cleave specific RNA sequences, while the first ("releasing") enzymatic DNA molecule has nuclease activity allowing it to liberate the "released" molecules. In another embodiment, the "released" enzymatic DNA molecule has amide bond-cleaving activity, while the first ("releasing") enzymatic DNA molecule has nuclease activity.

Alternatively, the first enzymatic DNA molecule may be encoded on a separate vector from the second (and third, fourth, etc.) enzymatic DNA molecule(s) and may have intermolecular cleaving activity. As noted herein, the first enzymatic DNA molecule can be a self-cleaving enzymatic DNA molecule (e.g., a deoxyribozyme), and the second enzymatic DNA molecule may be any desired type of enzymatic DNA molecule. When a vector is caused to express DNA from these nucleic acid sequences, that DNA has the ability under appropriate conditions to cleave each of the flanking regions, thereby releasing one or more copies of the second enzymatic DNA molecule. If desired, several different second enzymatic DNA molecules can be placed in the same cell or carrier to produce different deoxyribozymes. It is also contemplated that any one or more vectors may comprise one or more ribozymes or deoxyribozymes in any combination of "releasing" and "released" enzymatic nucleic acid molecules, as long as such a combination achieves the desired result: the release of enzymatic nucleic acid molecules that are capable of

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cleaving predetermined nucleic acid sequences.

Methods of isolating and purifying enzymatic DNA molecules of the present invention are also contemplated. In addition to the methods described herein, various purification methods (e.g. those using HPLC) and chromatographic isolation techniques are available in the art. See, e.g., the methods described in published international application no. WO 93/23569, the disclosures of which are incorporated herein by reference.

It should also be understood that various combinations of the embodiments described herein are included within the scope of the present invention. Other features and advantages of the present invention will be apparent from the descriptions hereinabove, from the Examples to follow, and from the claims.

#### EXAMPLES

The following examples illustrate, but do not limit, the present invention.

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1. In Vitro Evolution of Enzymatic DNA Molecules:An Overview

*In vitro* selection and *in vitro* evolution techniques allow new catalysts to be isolated without a priori knowledge of their composition or structure. Such methods have been used to obtain RNA enzymes with novel catalytic properties. For example, ribozymes that undergo autolytic cleavage with lead cation have been derived from a randomized pool of tRNA<sup>Phe</sup> molecules (Pan et al, *Biochemistry*, 31:3887-3895, 1992). Group I ribozyme variants have been isolated that can cleave DNA (Beaudry et al, *Science*, 257:635-641, 1992) or that have altered metal dependence (Lehman et al, *Nature*, 361:182-185, 1993).

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Starting with a pool of random RNA sequences, molecules have been obtained that catalyze a polymerase-like reaction (Bartel et al, Science, 261:1411-1418, 1993). In the present example, refinement of specific catalytic properties 5 of an evolved enzyme via alteration of the selection constraints during an *in vitro* evolution procedure is described.

Darwinian evolution requires the repeated operation of three processes: (a) introduction of genetic variation; (b) 10 selection of individuals on the basis of some fitness criterion; and (c) amplification of the selected individuals. Each of these processes can be realized *in vitro* (Joyce, Gene, 82:83, 1989). A gene can be mutagenized by chemical modification, incorporation of randomized 15 mutagenic oligodeoxynucleotides, or inaccurate copying by a polymerase. (See Cadwell et al, PCR Methods and Applications, 2:28-33, 1992); Cadwell et al, PCR Methods and Applications, 3(Suppl.):S136-S140, 1994; Chu et al, Virology, 98:168, 1979; Shortle et al, Meth. Enzymol., 20 100:457, 1983; Myers et al, Science, 229:242, 1985; Matteucci et al, Nucleic Acids Res., 11:3113, 1983; Wells et al, Gene, 34:315, 1985; McNeil et al, Mol. Cell. Biol., 25 5:3545, 1985; Hutchison et al, Proc.Natl.Acad.Sci. USA, 83:710, 1986; Derbyshire et al, Gene, 46:145, 1986; Zakour et al, Nature, 295:708, 1982; Lehtovaara et al, Protein Eng., 2:63, 1988; Leung et al, Technique, 1:11, 1989; Zhou et al, Nucl. Acids Res., 19:6052, 1991).

The gene product can be selected, for example, by its ability to bind a ligand or to carry out a chemical 30 reaction. (See, e.g., Joyce, Id., 1989; Robertson et al, Nature, 344:467, 1990; Tuerk et al, Science, 249:505, 1990). The gene that corresponds to the selected gene product can be amplified by a reciprocal primer method, such

as the polymerase chain reaction (PCR). (See Saiki et al, Science, 230:1350-54, 1985; Saiki et al, Science, 239:487-491, 1988).

Alternatively, nucleic acid amplification may be  
5 carried out using self-sustained sequence replication (3SR).  
(See Guatelli et al, Proc.Natl.Acad.Sci USA, 87:1874, 1990,  
the disclosures of which are incorporated by reference  
herein.) According to the 3SR method, target nucleic acid  
sequences may be amplified (replicated) exponentially in  
10 vitro under isothermal conditions by using three enzymatic  
activities essential to retroviral replication: (1) reverse  
transcriptase, (2) RNase H, and (3) a DNA-dependent RNA  
polymerase. By mimicking the retroviral strategy of RNA  
replication by means of cDNA intermediates, this reaction  
15 accumulates cDNA and RNA copies of the original target.

In summary, if one is contemplating the evolution of a  
population of enzymatic DNA molecules, a continuous series  
of reverse transcription and transcription reactions  
replicates an RNA target sequence by means of cDNA  
20 intermediates. The crucial elements of this design are (a)  
the oligonucleotide primers both specify the target and  
contain 5' extensions encoding the T7 RNA polymerase binding  
site, so that the resultant cDNAs are competent  
transcription templates; (b) cDNA synthesis can proceed to  
25 completion of both strands due to the degradation of  
template RNA in the intermediate RNA-DNA hybrid by RNase H;  
and (c) the reaction products (cDNA and RNA) can function as  
templates for subsequent steps, enabling exponential  
replication.

If one is evolving enzymatic DNA molecules, various  
critical elements of this design are somewhat different, as  
disclosed in these Examples. For instance, (1) the  
oligonucleotide primers specify the target and are

preferably "marked" or labeled in some fashion -- e.g., via biotinylation -- so the resultant competent template strands are easily identified; and (2) the *in vitro* selection procedure used preferably depends upon the identification of 5 the most favorable release mechanism.

A major obstacle to realizing Darwinian evolution *in vitro* is the need to integrate mutation and amplification, both of which are genotype-related, with selection, which is phenotype-related. In the case of nucleic acid enzymes, for 10 which genotype and phenotype are embodied in the same molecule, the task is simplified.

#### A. Design of Enzymatic DNA Molecules

It is well known that single-stranded DNA can 15 assume interesting tertiary structures. The structure of a "tDNA", for example, closely resembles that of the corresponding tRNA. (See Paquette et al, Eur. J. Biochem., 189:259-265, 1990). Furthermore, it has been possible to replace as many as 31 of 35 ribonucleotides within a 20 hammerhead ribozyme, while retaining at least some catalytic activity. (See Perreault et al, Nature, 344:565-567, 1990; Williams et al, Proc. Natl. Acad. Sci. USA, 89:918-921, 1992; Yang et al, Biochemistry, 31:5005-5009, 1992).

*In vitro* selection techniques have been applied to 25 large populations of random-sequence DNAs, leading to the recovery of specific DNA "aptamers" that bind a target ligand with high affinity (Bock et al, Nature, 355:564-566, 1992); Ellington et al Nature, 355:850-852, 1992; Wyatt et al, Proc.Natl.Acad.Sci. USA, 91:1356-1360, 1994). Recently, 30 two groups carried out the first NMR structural determination of an aptamer, a 15mer DNA that forms a G-quartet structure and binds the protein thrombin with high affinity (Wang et al, Biochemistry, 32:1899-1904, 1993;

Macaya et al, Proc.Natl.Acad.Sci. USA, 90:3745-3749, 1993). These findings were corroborated by an X-ray crystallographic analysis (Padmanabhan et al, J. Biol. Chem., 268:17651-17654, 1993).

5       The ability to bind a substrate molecule with high affinity and specificity is a prerequisite of a good enzyme. In addition, an enzyme must make use of well-positioned functional groups, either within itself or a cofactor, to promote a particular chemical transformation. Furthermore,  
10      the enzyme must remain unchanged over the course of the reaction and be capable of operating with catalytic turnover. Some would add the requirement that it be an informational macromolecule, comprised of subunits whose specific ordering is responsible for catalytic activity.  
15      While these criteria are open to debate on both semantic and chemical grounds, they serve to distinguish phenomena of chemical rate enhancement that range from simple solvent effects to biological enzymes operating at the limit of substrate diffusion (Albery et al, Biochemistry,  
20      15:5631-5640, 1976)..

As described in greater detail hereinbelow, we sought to develop a general method for rapidly obtaining DNA catalysts and DNA enzymes, starting from random sequences. As an initial target, we chose a reaction that we felt was well within the capability of DNA: the hydrolytic cleavage of an RNA phosphodiester, assisted by a divalent metal cofactor. This is the same reaction that is carried out by a variety of naturally-occurring RNA enzymes, including the hammerhead and hairpin motifs. (See, e.g., Forster et al, Cell, 49:211-220, 1987; Uhlenbeck, Nature, 328:596-600, 1987; Hampel et al, Biochemistry, 28:4929-4933, 1989).

It has recently been shown that, beginning with a randomized library of tRNA molecules, one can obtain

ribozymes that have Pb<sup>2+</sup>-dependent, site-specific RNA phosphoesterase activity at neutral pH (Pan et al, Biochemistry, 31:3887-3895, 1992; Pan et al, Nature, 358:560-563, 1992). This is analogous to the fortuitous self-cleavage reaction of yeast tRNA<sup>Phe</sup> (Dirheimer et al, Biochimie, 54:127-144, 1972), which depends on specific coordination of a Pb<sup>2+</sup> ion at a defined site within the tRNA. (See Rubin et al, J. Biomol. Struct. Dyn., 1:639-646, 1983; Brown et al, Biochemistry, 24:4785-4801, 1985).

As disclosed herein, our goals included the development of DNAs that could carry out Pb<sup>2+</sup>-dependent cleavage of a particular RNA phosphoester, initially presented within a short leader sequence attached to the 5' end of the DNA, and ultimately located within a separate molecule that could be cleaved in an intermolecular fashion with rapid catalytic turnover. These goals were successfully achieved, as described further below.

No assumptions were made as to how the DNA would interact with the target phosphoester and surrounding nucleotides. Beginning with a pool of approximately 10<sup>14</sup> random 50mer sequences, *in vitro* selection was allowed to run its course. After five rounds of selection carried out over four days, the population as a whole had attained the ability to cleave the target phosphoester in the presence of 1 mM Pb<sup>2+</sup> at a rate of about 0.2 min<sup>-1</sup>. This is an approximately 10<sup>5</sup>-fold increase compared to the spontaneous rate of cleavage under the same reaction conditions.

Individuals were isolated from the population, sequenced, and assayed for catalytic activity. Based on this information, the reaction was converted to an intermolecular format and then simplified to allow site-specific cleavage of a 19mer substrate by a 38mer DNA enzyme, in a reaction that proceeds with a turnover rate of

1 min<sup>-1</sup> at 23°C and pH 7.0 in the presence of 1 mM PbOAc.

B. In Vitro Selection Scheme

A starting pool of approximately 10<sup>14</sup> single-stranded DNA molecules was generated, all of which contain a 5' biotin moiety, followed successively by a fixed domain that includes a single ribonucleotide, a potential catalytic domain comprised of 50 random deoxyribonucleotides, and a second fixed domain that lay at the 3' terminus (Figure 1).

The pool was constructed by a nested PCR (polymerase chain reaction) technique, beginning with synthetic DNA that contained 50 random nucleotides flanked by primer binding sites. The nested PCR primer was a 5'-biotinylated synthetic oligodeoxynucleotide with a 3'-terminal adenosine ribonucleotide. Ribonucleotide-terminated oligonucleotides efficiently prime template-directed elongation in the context of the PCR, in this case giving rise to an extension product that contains a single embedded ribonucleotide.

Figure 1 illustrates a selective amplification scheme for isolation of DNAs that cleave a target RNA phosphoester. Double-stranded DNA containing a stretch of 50 random nucleotides is amplified via PCR, employing a 5'-biotinylated DNA primer (e.g., primer 3 -- 3a or 3b) terminated at the 3' end by an adenosine ribonucleotide (represented by the symbol "N" or "rA", wherein both N and rA represent an adenosine ribonucleotide). This primer is extended by Taq polymerase to yield a DNA product that contains a single embedded ribonucleotide. The resulting double-stranded DNA is immobilized on a streptavidin matrix and the unbiotinylated DNA strand is removed by washing with 0.2 N NaOH. After re-equilibrating the column with a buffered solution, the column is washed with the same solution with added 1 mM PbOAc. DNAs that undergo

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Pb<sup>2+</sup>-dependent self-cleavage are released from the column, collected in the eluant, and amplified by PCR. The PCR products are then used to initiate the next round of selective amplification.

5       The PCR products were passed over a streptavidin affinity matrix, resulting in noncovalent attachment of the 5'-biotinylated strand of the duplex DNA. The nonbiotinylated strand was removed by brief washing with 0.2 N NaOH, and the bound strand was equilibrated in a buffer containing 0.5 M NaCl, 0.5 M KCl, 50 mM MgCl<sub>2</sub>, and 50 mM HEPES (pH 7.0) at 23°C. Next, 1 mM PbOAc was provided in the same buffer, allowing Pb<sup>2+</sup>-dependent cleavage to occur at the target phosphoester, thereby releasing a subset of the DNAs from the streptavidin matrix. In principle, an individual DNA might facilitate its own release by various means, such as disruption of the interaction between biotin and streptavidin or cleavage of one of the deoxyribonucleotide linkages. It was felt that cleavage of the ribonucleoside 3'-O-P bond would be the most likely mechanism for release, based on the relative lability of this linkage, and that Pb<sup>2+</sup>-dependent hydrolytic cleavage would allow release to occur most rapidly. In principle, however, the *in vitro* selection procedure should identify the most favorable release mechanism as well as those individuals best able to carry out that mechanism.

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DNA molecules released from the matrix upon addition of Pb<sup>2+</sup> were collected in the eluant, concentrated by precipitation with ethanol, and subjected to nested PCR amplification. As in the construction of the starting pool of molecules, the first PCR amplification utilized primers that flank the random region (primers 1 and 2) and the second utilized a 5'-biotinylated primer (primer 3b) that has a 3'-terminal riboadenylate, thereby reintroducing the

30

target RNA phosphoester. The entire selective amplification procedure requires 3-4 hours to perform.

The molecules are purified in three ways during each round of this procedure: first, following PCR amplification, by extracting twice with phenol and once with chloroform / isoamyl alcohol, then precipitating with ethanol; second, following attachment of the DNA to streptavidin, by washing away all the nonbiotinylated molecules under strongly denaturing conditions; and third, following elution with  $Pb^{2+}$ , by precipitating with ethanol. There is no gel electrophoresis purification step, and thus no selection pressure constraining the molecules to a particular length.

C. Selection of Catalytic DNA

Five successive rounds of *in vitro* selection were carried out, progressively decreasing the reaction time following addition of  $Pb^{2+}$  in order to progressively increase the stringency of selection. During rounds 1 through 3, the reaction time was 1 hour; during round 4, the reaction time was 20 minutes; and during round 5, it was 1 minute. The starting pool of single-stranded DNAs, together with the population of molecules obtained after each round of selection, was assayed for self-cleavage activity under conditions identical to those employed during *in vitro* selection (see Figure 2).

For this assay, the molecules were prepared with a 5'- $^{32}P$  rather than a 5'-biotin moiety, allowing detection of both the starting material and the 5' cleavage product. Following a 5-minute incubation, there was no detectable activity in the initial pool (G0) or in the population obtained after the first and second rounds of selection. DNAs obtained after the third round (G3) exhibited a modest level of activity; this activity increased steadily,

reaching approximately 50% self-cleavage for the DNAs obtained after the fifth round of selection (G5). Cleavage was detected only at the target phosphoester, even after long incubation times. This activity was lost if Pb<sup>2+</sup> was  
5 omitted from the reaction mixture.

Figure 2 illustrates the self-cleavage activity of the starting pool of DNA (G0) and populations obtained after the first through fifth rounds of selection (G1 - G5). Reaction mixtures contained 50 mM MgCl<sub>2</sub>, 0.5 M NaCl, 0.5 M KCl, 50 mM  
10 HEPES (pH 7.0 at 23°C), and 3 nM [5'-<sup>32</sup>P]-labeled DNA, incubated at 23°C for 5 min either in the presence or in the absence of 1 mM PbOAc. The symbol Pre represents  
15 108-nucleotide precursor DNA (SEQ ID NO 4); Clv,  
28-nucleotide 5'-cleavage product (SEQ ID NO 5); and M,  
primer 3a (SEQ ID NO 6), corresponding in length to the  
5'-cleavage product.

The 28-nucleotide 5' cleavage product (Clv) illustrated preferably has the sequence 5'-GGGACGAATTCTAATACGACTCACTATN-  
20 3', wherein "N" represents adenosine ribonucleotide with an additional 2', 3'-cyclic phosphate on the 3' end (SEQ ID NO 5). In alternative embodiments, "N" represents adenosine ribonucleotide with an additional 2' or 3' phosphate on the  
3' end of the molecule.

In Figure 2, the "G0" lane "Pre" band comprises a  
25 sampling of 108-nucleotide precursor DNAs that each include 50 random nucleotides. Therefore, any given "Pre" sampling will contain a wide variety of precursor DNAs, and each sampling will likely differ from previous and subsequent samplings. The "G1" through "G5" lanes contain "Pre" bands  
30 that are increasingly enriched for catalytic DNA molecules, but still contain a large number of different DNA sequences (i.e., differing in the 50 nucleotide randomized domain). A sample of these different sequences from "G5 Pre" DNA is

provided in Figure 3.

Shotgun cloning techniques were employed to isolate individuals from the G5 population; the complete nucleotide sequences of 20 of these subclones were then determined (see 5 Figure 3). (Also see Cadwell et al, PCR Methods and Applications, 2:28-33, 1992); and Cadwell et al, PCR Methods and Applications, 3(Suppl.):S136-S140, 1994). Of the 20 sequences, five were unique, two occurred twice, one occurred three times, and one occurred eight times. All of 10 the individual variants share common sequence elements within the 50-nucleotide region that had been randomized in the starting pool of DNA. They all contain two presumed template regions, one with complementarity to a stretch of nucleotides that lies just upstream from the cleavage site 15 and the other with complementarity to nucleotides that lie at least four nucleotides downstream. Between these two presumed template regions lies a variable domain of 1-11 nucleotides, followed by the fixed sequence 5'-AGCG-3', then a second variable domain of 3-8 nucleotides, and finally the 20 fixed sequence 5'-CG-3' or 5'-CGA-3'. Nucleotides that lie outside of the two presumed template regions are highly 25 variable in both sequence and length. In all of the sequenced subclones, the region corresponding to the 50 initially-randomized nucleotides remains a total of 50 nucleotides in length.

Figure 3 illustrates the sequence alignment of individual variants isolated from the population after five rounds of selection. The fixed substrate domain (5'-GGGACGAATTCTAATACGACTCACTATrAGGAAGAGATGGCGAC-3', or 30 5'-GGGACGAATTCTAATACGACTCACTATNGGAAGAGATGGCGAC-3', where N represents adenine ribonucleotide) (SEQ ID NO 13) is shown at the top, with the target riboadenylate identified with an inverted triangle. Substrate nucleotides that are commonly

involved in presumed base-pairing interactions are indicated by a vertical bar. Sequences corresponding to the 50 initially-randomized nucleotides are aligned antiparallel to the substrate domain. All of the variants are 3'-terminated  
5 by the fixed sequence 5'-CGGTAAGCTTGGCAC-3' (SEQ ID NO 1) ("primer site"; not shown). Nucleotides within the initially-randomized region that are presumed to form base pairs with the substrate domain are indicated on the right and left sides of the Figure; the putative base-pair-forming  
10 (or substrate binding) regions of the enzymatic DNA molecules are individually boxed in each sequence shown. The highly-conserved nucleotides within the putative catalytic domain are illustrated in the two boxed columns.

15 While it is anticipated that additional data will be helpful in constructing a meaningful secondary structural model of the catalytic domain, we note that, like the hammerhead and hairpin ribozymes, the catalytic domain of our enzymatic DNA molecules appears to contain a conserved core flanked by two substrate binding regions (or  
20 recognition domains) that interact with the substrate through base-pairing interactions. Similar to the hammerhead and hairpin ribozymes, the catalytic DNAs also appear to require a short stretch of unpaired substrate nucleotides -- in this case 5'-GGA-3' -- between the two  
25 regions that are involved in base pairing.

It was also interesting to note that each of the nine distinct variants exhibited a different pattern of presumed complementarity with the substrate domain. In some cases, base pairing was contiguous, while in others it was  
30 interrupted by one or more noncomplementary pairs. The general tendency seems to be to form tighter interaction with the nucleotides that lie upstream from the cleavage site compared to those that lie downstream. Binding studies

and site-directed mutagenesis analysis should enable us to gain further insights and to further substantiate this conjecture.

In order to gain further insight into the sequence requirements for catalytic function, the self-cleavage activity of six of the nine variants was tested and evaluated under the within-described selection conditions (see Figure 3). Not surprisingly, the sequence that occurred in eight of the 20 subclones proved to be the most reactive, with a first-order rate constant of  $1.4 \text{ min}^{-1}$ . All of the studied variants were active in the self-cleavage assay and all gave rise to a single 5'-labeled product corresponding to cleavage at the target RNA phosphoester.

The dominant subclone was further analyzed under a variety of reaction conditions. Its self-cleavage activity was dependent on  $\text{Pb}^{2+}$  but was unaffected if  $\text{Mg}^{2+}$  was omitted from the reaction mixture. There was a requirement for a monovalent cation as well, which can be met by either  $\text{Na}^+$  or  $\text{K}^+$ . The reaction rate increased linearly with increasing concentration of monovalent cation over the range of 0 - 1.0 M ( $r = 0.998$ ). Other variables that may affect the reaction, such as pH, temperature, and the presence of other divalent metals, are in the process of being evaluated further.

25

## 2. Materials and Methods

### A. Oligonucleotides and Oligonucleotide Analogs

Synthetic DNAs and DNA analogs were purchased from Operon Technologies. The 19-nucleotide substrate, 5'-pTCACTATrAGGAAGAGATGG-3' (or 5'-pTCACTATNGGAAGAGATGG-3', wherein "N" represents adenosine ribonucleotide) (SEQ ID NO 7), was prepared by reverse-transcriptase catalyzed extension of 5'-pTCACTATrA-3' (or 5'-pTCACTATN-3', wherein

"N" represents adenosine ribonucleotide) (SEQ ID NO 8), as previously described (Breaker et al, Biochemistry, 33:11980-11986, 1994), using the template 5'-CCATCTTCCCTATAGTGAGTCGGCTGCA-3' (SEQ ID NO 9). Primer 5, 5'-GGGACGAATTCTAATACGACTCACTATrA-3' (or 5'-GGGACGAATTCTAATACGACTCACTATN-3', wherein "N" represents adenosine ribonucleotide) (SEQ ID NO 6), was either 5'-labeled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase (primer 3a) or 5'-thiophosphorylated with [ $\gamma$ -S]ATP and T4 10 polynucleotide kinase and subsequently biotinylated with N-iodoacetyl-N'-biotinylhexylenediamine (primer 3b).

B. DNA Pool Preparation

The starting pool of DNA was prepared by PCR using 15 the synthetic oligomer 5'-GTGCCAAGCTTACCG-N<sub>50</sub>-GTCGCCATCTCTCC-3' (SEQ ID NO 4), where N is an equimolar mixture of G, A, T and C. A 2-ml PCR, containing 500 pmoles of the randomized oligomer, 1,000 pmoles primer 1 (5'-GTGCCAAGCTTACCG-3', SEQ ID NO 10), 500 20 pmoles primer 2 (5'-CTGCAGAATTCTAATACGACTCACTATAAGGAAGAGATGGCGAC-3', SEQ ID NO 11), 500 pmoles primer 3b, 10  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dATP, and 0.2 U  $\mu$ l<sup>-1</sup> Tag DNA polymerase, was incubated in the presence of 50 25 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.3 at 23°C), 0.01% gelatin, and 0.2 mM of each dNTP for 1 min at 92°C, 1 min at 50°C, and 2 min at 72°C, then 5 cycles of 1 min at 92°C, 1 min at 50°C, and 1 min at 72°C. The resulting mixture was extracted twice with phenol and once with chloroform / isoamyl alcohol, and the DNA was isolated by precipitation 30 with ethanol.

C. In Vitro Selection

The starting pool of DNA was resuspended in 500  $\mu$ L

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of buffer A (1 M NaCl and 50 mM HEPES (pH 7.0 at 23°C)) and was passed repeatedly over a streptavidin column (AffiniTip Strep 20, Genosys, The Woodlands, TX). The column was washed with five 100- $\mu$ l volumes of buffer A, followed by 5 five 100- $\mu$ l volumes of 0.2 N NaOH, then equilibrated with five 100- $\mu$ l volumes of buffer B (0.5 M NaCl, 0.5 M KCl, 50 mM MgCl<sub>2</sub>, and 50 mM HEPES (pH 7.0 at 23°C)). The immobilized single-stranded DNA was eluted over the course of 1 hr with three 20- $\mu$ l volumes of buffer B with added 1 mM PbOAc. The entire immobilization and elution process was conducted at 23°C. The eluant was collected in an equal volume of buffer C (50 mM HEPES (pH 7.0 at 23°C) and 80 mM EDTA) and the DNA was precipitated with ethanol.

The resulting DNA was amplified in a 100- $\mu$ L PCR containing 20 pmoles primer 1, 20 pmoles primer 2, 0.05 U  $\mu$ l<sup>-1</sup> Taq polymerase, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH 8.3 at 23°C), 0.01% gelatin, and 0.2 mM of each dNTP for 30 cycles of 10 sec at 92°C, 30 sec at 50°C, and 30 sec at 72°C. The reaction products were extracted twice with phenol and once with chloroform / isoamyl alcohol, and the DNA was recovered by precipitation with ethanol. Approximately 4 pmoles of the amplified DNA was added to a second, nested PCR containing 100 pmoles primer 1, 100 pmoles primer 3b, 20  $\mu$ Ci [ $\alpha$ -<sup>32</sup>P]dATP, and 0.1 U  $\mu$ l<sup>-1</sup> Taq polymerase, in a total volume of 200  $\mu$ L that was amplified for 10 cycles of 1 min at 92°C, 1 min at 50°C, and 1 min at 72°C. The PCR products were once more extracted and precipitated, and the resulting DNA was resuspended in 50  $\mu$ L buffer A, then used to begin the next round of selection.

30 The second and third rounds were carried out as above, except that the nested PCR at the end of the third round was performed in a 100- $\mu$ l volume. During the fourth round, the elution time following addition of Pb<sup>2+</sup> was reduced to 20

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min (two 20- $\mu$ L elution volumes) and only half of the recovered DNA was used in the first PCR, which involved only 15 temperature cycles. During the fifth round, the elution time was reduced to 1 min (two 20- $\mu$ L elution volumes) and only one-fourth of the recovered DNA was used in the first PCR, which involved 15 temperature cycles. DNA obtained after the fifth round of selection was subcloned and sequenced, as described previously (Tsang et al, *Biochemistry*, 33:5966-5973, 1994).

10

D. Kinetic Analysis of Catalytic DNAs

Populations of DNA and various subcloned individuals were prepared with a 5'- $^{32}$ P label by asymmetric PCR in a 25- $\mu$ l reaction mixture containing 10 pmoles primer 3a, 0.5 pmoles input DNA, and 0.1 U  $\mu$ l $^{-1}$  Taq polymerase, under conditions as described above, for 10 cycles of 1 min at 92°C, 1 min at 50°C, and 1 min at 72°C. The resulting [5'- $^{32}$ P]-labeled amplification products were purified by electrophoresis in a 10% polyacrylamide / 8 M gel.

15

Self-cleavage assays were carried out following preincubation of the DNA in buffer B for 10 min. Reactions were initiated by addition of PbOAc to 1 mM final concentration and were terminated by addition of an equal volume of buffer C. Reaction products were separated by 20 electrophoresis in a 10% polyacrylamide / 8 M gel. Kinetic assays under multiple-turnover conditions were carried out 25 in buffer B that included 50  $\mu$ g ml $^{-1}$  BSA to prevent adherence of material to the vessel walls. Substrate and enzyme molecules were preincubated separately for 5 min in reaction buffer that lacked Pb $^{2+}$ , then combined, and the 30 reaction was initiated by addition of PbOAc to a final concentration of 1 mM.

3. Evolution of Deoxyribozymes That Cleave  
Intermolecularly

A. Conversion to an Intermolecular Format

Based on the variable pattern of presumed  
5 base-pairing interactions between the catalytic and  
substrate domains of the studied variants, it was felt that  
it would be reasonably straightforward to convert the  
DNA-catalyzed reaction to an intermolecular format. In  
doing so, we wished to simplify the two substrate-binding  
10 regions of the catalyst so that each would form an  
uninterrupted stretch of 7-8 base pairs with the substrate.  
In addition, we wished to provide a minimal substrate,  
limited to the two base-pairing regions and the intervening  
sequence 5'-GGA-3' (Figure 4A).

15 Figures 4A and 4B illustrate DNA-catalyzed cleavage of  
an RNA phosphoester in an intermolecular reaction that  
proceeds with catalytic turnover. Figure 4A is a  
diagrammatic representation of the complex formed between  
the 19mer substrate and 38mer DNA enzyme. The substrate  
20 contains a single adenosine ribonucleotide ("rA" or "N",  
adjacent to the arrow), flanked by deoxyribonucleotides.  
The synthetic DNA enzyme is a 38-nucleotide portion of the  
most frequently occurring variant shown in Figure 3.  
Highly-conserved nucleotides located within the putative  
25 catalytic domain are "boxed". As illustrated, one conserved  
sequence is "AGCG", while another is "CG" (reading in the  
5'-3' direction).

Figure 4B shows an Eadie-Hofstee plot used to determine  
K<sub>m</sub> (negative slope) and V<sub>max</sub> (y-intercept) for DNA-catalyzed  
30 cleavage of [5'-<sup>32</sup>P]-labeled substrate under conditions  
identical to those employed during *in vitro* selection.  
Initial rates of cleavage were determined for reactions  
involving 5 nM DNA enzyme and either 0.125, 0.5, 1, 2, or 4

$\mu$ M substrate.

In designing the catalytic domain, we relied heavily on the composition of the most reactive variant, truncating by two nucleotides at the 5' end and 11 nucleotides at the 3' end. The 15 nucleotides that lay between the two template regions were left unchanged and a single nucleotide was inserted into the 3' template region to form a continuous stretch of nucleotides capable of forming base pairs with the substrate. The substrate was simplified to the sequence 5'-TCACTATrA • GGAAGAGATGG-3' (or 5'-TCACTATN • GGAAGAGATGG-3', wherein "N" represents adenosine ribonucleotide) (SEQ ID NO 12), where the underlined nucleotides correspond to the two regions involved in base pairing with the catalytic DNA molecule.

The simplified reaction system, employing a 38mer catalytic DNA molecule (catalyst) comprised entirely of deoxyribonucleotides and a 19mer substrate containing a single ribonucleotide embedded within an otherwise all-DNA sequence, allows efficient DNA-catalyzed phosphoester cleavage with rapid turnover. Over a 90-minute incubation in the presence of 0.01  $\mu$ M catalyst and 1  $\mu$ M substrate, 46% of the substrate is cleaved, corresponding to 46 turnovers of the catalyst. A preliminary kinetic analysis of this reaction was carried out, evaluated under multiple-turnover conditions. The DNA catalyst exhibits Michaelis-Menten kinetics, with values for  $k_{cat}$  and  $K_m$  of 1  $\text{min}^{-1}$  and 2  $\mu\text{M}$ , respectively (see Figure 4B). The value for  $K_m$  is considerably greater than the expected dissociation constant between catalyst and substrate based on Watson-Crick interactions. The substrate was incubated under identical reaction conditions (but in the absence of the catalyst); a value for  $k_{uncat}$  of  $4 \times 10^{-6} \text{ min}^{-1}$  was obtained. This is consistent with the reported value of  $5 \times 10^{-3} \text{ min}^{-1}$  for

hydrolysis of the more labile 1-nitrophenyl-1,2-propanediol in the presence of 0.5 mM Pb<sup>2+</sup> at pH 7.0 and 37°C (Breslow et al, Proc.Natl.Acad.Sci. USA, 88:4080-4083, 1991).

It is now presumed that the phosphoester cleavage reaction proceeds via a hydrolytic mechanism involving attack by the ribonucleoside 2'-hydroxyl on the vicinal phosphate, generating a 5' product with a terminal 2'(3')-cyclic phosphate and 3' product with a terminal 5'-hydroxyl. In support of this mechanism, the 3'-cleavage product is efficiently phosphorylated with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP, consistent with the availability of a free 5'-hydroxyl.

B. Discussion

After five rounds of *in vitro* selection, a population of single-stranded DNA molecules that catalyze efficient Pb<sup>2+</sup>-dependent cleavage of a target RNA phosphoester was obtained. Based on the common features of representative individuals isolated from this population, a simplified version of both the catalytic and substrate domains was constructed, leading to a demonstration of rapid catalytic turnover in an intermolecular context. Thus the 38mer catalytic domain provides an example of a DNA enzyme, or what might be termed a "deoxyribozyme".

Referring to this molecule as an enzyme, based on the fact that it is an informational macromolecule capable of accelerating a chemical transformation in a reaction that proceeds with rapid turnover and obeys Michaelis-Menten kinetics, may not satisfy everyone's notion of what constitutes an enzyme. Some might insist that an enzyme, by definition, must be a polypeptide. If, however, one accepts the notion of an RNA enzyme, then it seems reasonable to adopt a similar view concerning DNA enzymes. Considering

how quickly we were able to generate this molecule from a pool of random-sequence DNAs, we expect that many other examples of synthetic DNA enzymes will appear in the near future.

5       The Pb<sup>2+</sup>-dependent cleavage of an RNA phosphoester was chosen as an initial target for DNA catalysis because it is a straightforward reaction that simply requires the proper positioning of a coordinated Pb<sup>2+</sup>-hydroxyl to facilitate deprotonation of the 2' hydroxyl that lies adjacent to the  
10      cleavage site. (See Pan et al, in The RNA World, Gesteland & Atkins (eds.), pp. 271-302, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1993).) Pb<sup>2+</sup> is known to coordinate to the N7 position of purines, the O6 position of guanine, the O4 position of uracil, and the N3 position of  
15      cytosine (Brown et al, Nature, 303:543-546, 1993). Thus, the differences in sugar composition and conformation of DNA compared to RNA seemed unlikely to prevent DNA from forming a well-defined Pb<sup>2+</sup>-binding pocket.

20      A substrate that contains a single ribonucleotide within an otherwise all-DNA sequence was chosen because it provided a uniquely favored site for cleavage and insured that any resulting catalytic activity would be attributable solely to DNA. Substrate recognition appears to depend on two regions of base-pairing interactions between the  
25      catalyst and substrate. However, the unpaired substrate nucleotides, 5'-GGA-3', that lie between these two regions may play an important role in substrate recognition, metal coordination, or other aspects of catalytic function.

30      It is further anticipated that an all-RNA molecule, other RNA-DNA composites, and molecules containing one or more nucleotide analogs may be acceptable substrates. As disclosed herein, the within-described *in vitro* evolution procedures may successfully be used to generate enzymatic

DNA molecules having the desired specificities; further analyses along these lines are presently underway.

In addition, studies to determine whether the presumed base-pairing interactions between enzyme and substrate are generalizable with respect to sequence are in progress, using the presently-described methods. The within-disclosed  $Pb^{2+}$ -dependent deoxyribozymes may also be considered model compounds for exploring the structural and enzymatic properties of DNA.

The methods employed in the present disclosure for the rapid development of DNA catalysts will have considerable generality, allowing us to utilize other cofactors to trigger the cleavage of a target linkage attached to a potential catalytic domain. In this regard, the development of  $Mg^{2+}$ -dependent DNA enzymes that specifically cleave target RNAs under physiological conditions is of interest, as is the development of DNA enzymes that function in the presence of other cations (see Example 4). Such molecules will provide an alternative to traditional antisense and ribozyme approaches for the specific inactivation of target mRNAs.

DNA thus joins RNA and protein on the list of biological macromolecules that are capable of exhibiting enzymatic activity. The full extent of DNA's catalytic abilities remains to be explored, but these explorations should proceed rapidly based on *in vitro* selection methods such as those employed in this study.

DNA enzymes offer several important advantages compared to other macromolecular catalysts. First, they are easy to prepare, in an era when most laboratories have access to an automated DNA synthesizer and the cost of DNA phosphoramidites has become quite modest. Second, they are very stable compounds, especially compared to RNA, thus

facilitating their use in biophysical studies. Third, we expect that they can be adapted to therapeutic applications that at present make use of antisense DNAs that lack RNA-cleavage activity. *In vitro* selection could be carried 5 out with DNA analogs, including compounds that are nuclease resistant such as phosphorothioate-containing DNA, so long as these analogs can be prepared in the form of a deoxynucleoside 5'-triphosphate and are accepted as a substrate by a DNA-dependent DNA polymerase. Finally, DNA 10 enzymes offer a new window on our understanding of the macromolecular basis of catalytic function. It will be interesting, for example, to carry out comparative analyses of protein-, RNA-, and DNA-based enzymes that catalyze the same chemical transformation.

15

#### 4. Other Families of Catalytic DNAs

A starting pool of DNA was prepared by PCR essentially as described in Example 2.B. above, except that the starting pool of DNA comprised molecules containing 40 random 20 nucleotides. Thus, the starting pool of DNA described herein was prepared by PCR using the synthetic oligomer 5' GGG ACG AAT TCT AAT ACG ACT CAC TAT rA GG AAG AGA TGG CGA CAT CTC N<sub>40</sub>GT GAC GGT AAG CTT GGC AC 3' (SEQ ID NO 23), where N is an equimolar mixture of G, A, T and C, and where 25 the DNA molecules were selected for the ability to cleave the phosphoester following the target rA. (See Figure 6A, also.)

Selective amplification was carried out in the presence of either Pb<sup>2+</sup>, Zn<sup>2+</sup>, Mn<sup>2+</sup>, or Mg<sup>2+</sup>, thereby generating at least 30 four "families" of catalytic DNA molecules. As illustrated in Figure 5, catalytic DNA molecules demonstrating specific activity were generated in the presence of a variety of cations.

Figure 5 is a photographic representation showing a polyacrylamide gel demonstrating specific endoribonuclease activity of four families of selected catalytic DNAs. Selection of a Pb<sup>2+</sup>-dependent family of molecules was 5 repeated in a side-by-side fashion as a control. In each group of three lanes, the first lane shows the lack of activity of the selected population in the absence of the metal cation, the second lane shows the observed activity in the presence of the metal cation, and the third lane shows 10 the lack of activity of the starting pool (G0). At present, the order of reactivity is observed to be Pb<sup>2+</sup>>Zn<sup>2+</sup>>Mn<sup>2+</sup>>Mg<sup>2+</sup>, mirroring the pK<sub>a</sub> of the corresponding metal-hydroxide.

After either five (G5) or six (G6) rounds of selective amplification in the presence of the preselected divalent cation, the desired endonuclease activity was obtained. The 15 following description of selective amplification in the presence of Mg<sup>2+</sup> is intended to be exemplary.

Six rounds of *in vitro* selective amplification were carried out, following the method described in Example 2 20 hereinabove, except that the divalent metal used was 1 mM Mg<sup>2+</sup> rather than 1 mM Pb<sup>2+</sup>. (See also Breaker et al, Chem. & Biol., 1:223-229, 1994), incorporated by reference herein, which describes essentially the same procedure.)

Individual clones were isolated following the sixth 25 round, and the nucleotide sequence of 24 of these clones was determined. All of the sequences began with: 5' GGG ACG AAT TCT AAT ACG ACT CAC TAT rA GG AAG AGA TGG CGA CA (SEQ ID NO 23 from position 1 to 44) and ended with: CGG TAA GCT TGG CAC 3' (SEQ ID NO 23 from position 93 to 107).

The segment in the middle, corresponding to TCTC N<sub>40</sub> 30 GTGA (SEQ ID NO 23 from position 45 to 92) in the starting pool, varied as follows:

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- (13) CCG CCC ACC TCT TTT ACG AGC CTG TAC GAA ATA GTG  
CTC TTG TTA GTA T (SEQ ID NO 24)
- (5) TCT **C**TT CAG CGA TGC ACG CTT GTT TTA ATG TTG CAC  
CCA TGT TAG TGA (SEQ ID NO 25)
- 5 (2) TCT CAT CAG CGA TTG AAC CAC TTG GTG GAC AGA CCC  
ATG TTA GTG A (SEQ ID NO 26)
- (1) CCG CCC ACC TCT TTT ACG AGC CTG TAC GAA ATA GTG  
TTC TTG TTA GTA T (SEQ ID NO 27)
- 10 (1) CCG CCC ACC TCT TTT ACG AGC CTG TAC GAA ATA GTG  
CTC TCG TTA GTA T (SEQ ID NO 28)
- (1) TCT CAG ACT TAG TCC ATC ACA CTC TGT GCA TAT GCC  
TGC TTG ATG TGA (SEQ ID NO 29)
- (1) -CT CTC ATC TGC TAG CAC GCT CGA ATA GTG TCA GTC  
GAT GTG A (SEQ ID NO 30).

15

The initial number in parentheses indicates the number of clones having that particular sequence. Note that some mutations (highlighted in bold type) occurred at nucleotide positions other than those that were randomized initially.

20 The second sequence listed above (i.e., SEQ ID NO 25), which occurred in 5 of 24 clones, was chosen as a lead (i.e. principal) compound for further study. Its cleavage activity was measured in the presence of a 1 mM concentration of various divalent metals and 1 M NaCl at pH 7.0 and 23°C:

	metal	$k_{obs}$ (min <sup>-1</sup> )
	none	n.d.
30	Mg <sup>2+</sup>	2.3 x 10 <sup>-3</sup>
	Mn <sup>2+</sup>	6.8 x 10 <sup>-3</sup>
	Zn <sup>2+</sup>	4.2 x 10 <sup>-2</sup>
	Pb <sup>2+</sup>	1.1 x 10 <sup>-2</sup>

Thus, the lead compound is active in the presence of all four divalent metals, even though it was selected for activity in the presence of Mg<sup>2+</sup>. Conversely, DNA molecules that were selected for activity in the presence of Mn<sup>2+</sup>, Zn<sup>2+</sup>, or Pb<sup>2+</sup> did not show any activity in the presence of Mg<sup>2+</sup>.

In addition, the population of DNAs obtained after six rounds of *in vitro* selection in the presence of Mg<sup>2+</sup>, when prepared as all-phosphorothioate-containing DNA analogs, showed Mg<sup>2+</sup>-dependent cleavage activity at an observed rate of ~10<sup>-3</sup> min<sup>-1</sup>. The phosphorothioate-containing analogs were prepared enzymatically so as to have an R<sub>p</sub> configuration at each stereocenter. Such compounds are relatively resistant to degradation by cellular nucleases compared to unmodified DNA.

The lead compound was re-randomized at 40 nucleotide positions (underlined), introducing mutations at a frequency of 15% (5% probability of each of the three possible base substitutions). The re-randomized population was subjected to seven additional rounds of *in vitro* selection. During the last four rounds, molecules that were reactive in the presence of 1 mM Pb<sup>2+</sup> were removed from the population before the remainder were challenged to react in the presence of 1 mM Mg<sup>2+</sup>. Individual clones were isolated following the seventh round and the nucleotide sequence of 14 of these clones was determined. All of the sequences began with: 5' GGG ACG AAT TCT AAT ACG ACT CAC TAT rA GG AAG AGA TGG CGA CAT CTC (SEQ ID NO 23, from position 1 to 48), and ended with: GTG ACG GTA AGC TTG GCA C 3' (SEQ ID NO 23, from position 89 to 107).

The segment in the middle, corresponding to the 40 partially-randomized positions (N<sub>40</sub>, SEQ ID NO 23, from position 49 to 88), varied as follows:

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- (4) TAC AGC GAT TCA CCC TTG TTT **AAG** GGT TAC ACC CAT  
GTT A (SEQ ID NO 31)
- (2) ATC AGC GAT **TAA** CGC TTG TTT CAA TGT TAC ACC CAT  
GTT A (SEQ ID NO 32)
- 5 (2) TTC AGC GAT TAA CGC TTA TTT TAG CGT TAC ACC CAT  
GTT A (SEQ ID NO 33)
- (1) ATC AGC GAT TCA CCC TTG TTT **TAA** GGT TGC ACC CAT  
GTT A (SEQ ID NO 34)
- (1) ATC AGC GAT TCA CCC TTG TTT **AAG** CGT TAC ACC CAT  
10 GTT G (SEQ ID NO 35)
- (1) ATC AGC GAT TCA CCC TTG TTT **TAA** GGT TAC ACC CAT  
GTT A (SEQ ID NO 36)
- (1) ATC AGC GAT **TAA** CGC TTA TTT TAG CGT TAC ACC CAT  
GTT A (SEQ ID NO 37)
- 15 (1) ATC AGC GAT **TAA** CGC TTG TTT TAG TGT TGC ACC CAT  
GTT A (SEQ ID NO 38)
- (1) ATC AGC GAT **TAA** CGC TTA TTT TAG CAT TAC ACC CAT  
GTT A (SEQ ID NO 39).

20 The number in parentheses indicates the number of clones having that particular sequence. Nucleotides shown in bold are those that differ compared to the lead compound.

Formal analysis of the cleavage activity of these clones is ongoing. The population as a whole exhibits 25  $Mg^{2+}$ -dependent cleavage activity at an observed rate of  $\sim 10^{-2}$  min<sup>-1</sup>, with a comparable level of activity in the presence of  $Pb^{2+}$ .

Figures 6A and 6B provide two-dimensional illustrations 30 of a "progenitor" catalytic DNA molecule and one of several catalytic DNA molecules obtained via the selective amplification methods disclosed herein, respectively. Figure 6A illustrates an exemplary molecule from the starting pool, showing the overall configuration of the

molecules represented by SEQ ID NO 23. As illustrated, various complementary nucleotides flank the random ( $N_{40}$ ) region.

Figure 6B is a diagrammatic representation of one of the  $Mg^{2+}$ -dependent catalytic DNA molecules (or "DNAzymes") generated via the within-described procedures. The location of the ribonucleotide in the substrate nucleic acid is indicated via the arrow. (The illustrated molecule includes the sequence identified herein as SEQ ID NO 25, as well as "beginning" and "ending" sequences of SEQ ID NO 23.)

Endonuclease activity is continuing to be enhanced in each of the aforementioned "families" via *in vitro* evolution, as disclosed herein, so it is anticipated that enzymatic DNA molecules of increasingly desirable specificities may be generated successfully using the within-disclosed guidelines.

##### 5. Cleavage of Larger RNA Sequences

As an extension of the foregoing, we have developed DNA enzymes that cleave an all-RNA substrate, rather than a single ribonucleotide embedded within an otherwise all-DNA substrate as demonstrated above. (Also see Breaker et al, Chem. & Biol., 1:223-229, 1994); Breaker et al, Chem. & Biol., 2:655-660, 1995). As a target sequence, we chose a stretch of 12 highly-conserved nucleotides within the U5 LTR region of HIV-1 RNA, having the sequence 5' GUACUAGAGAU 3' (SEQ ID NO 49).

Following the methods described in the previous examples, we generated a pool of  $10^{14}$  DNA molecules that have the following composition:

5'- GGAAAA r(GUAACUAGAGAU) GGAAGAGATGGCGAC N<sub>50</sub>

CGGTAAGCTTGGCAC -3' (SEQ ID NO 50),

where N is an equimolar mixture of the deoxyribonucleotides

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G, A, T, and C, and where the sequence identified as "r(GUAACUAGAGAU)" is comprised of ribonucleotides. (Optionally, one may alter the initial 5' nucleotide sequence, e.g., by adding an additional dA residue to the 5 sequence preceding the ribonucleotide portion at the 5' end, thus causing the initial sequence to read "GGAAAAAA" and causing SEQ ID NO 50 to be 99 residues in length. Clearly, this is but one example of the modifications that may be made in order to engineer specific enzymatic DNA molecules, 10 as disclosed in detail herein.)

The initial library was generated by template-directed extension of 50 pmols of 5'-biotin-d(GGAAAAAA)r(GUAACUAGAGAU)d(GGAAGAGATGGCGAC)-3' on 100 pmols of 5'-GTGCCAAGCTTACCG-N50-GTCGCCATCTCTTCC-3' (N = G, A, T or C), in a 50-ul reaction mixture containing 10 U ul<sup>-1</sup> Superscript II reverse transcriptase (RT; Gibco BRL), 3 mM MgCl<sub>2</sub>, 75 mM KCl, 50 mM Tris\*HCl (pH 8.3), and 0.2 mM of each dNTP. A trace amount of [5'-32P]-labeled primer was included in the reaction mixture to allow extension 20 efficiency to be monitored. All components except RT were combined, incubated at 65 °C for 5 min, then cooled to 45 °C over 10 min. RT was added and the mixture was incubated at 45 °C for 45 min, then quenched by addition of Na<sub>2</sub> EDTA. NaCl was added to a final concentration of 1 M and the 25 extension products were immobilized by repeated passing through four streptavidin affinity columns (Genosys). The columns were washed with five 100-ul volumes of wash buffer (1 M NaCl, 50 mM Tris\*HCl (pH 7.5), 0.1 mM Na<sub>2</sub>EDTA), followed by five 100-ul volumes of 0.1 N NaOH and five 30 100-ul volumes of wash buffer at 37 °C, then eluted at 37 °C over 1 hr with three 20-ul aliquots of reaction buffer (10 mM MgCl<sub>2</sub>, 1 M NaCl, 50 mM Tris\*HCl (pH 7.5)). Eluted molecules were recovered and amplified by the polymerase

chain reaction (PCR) using the primers 5'-biotin-GGAAGAGATGGCGAC-3' and 5'-GTGCCAAGCTTACCG-3'. The PCR products were immobilized on streptavidin columns, as above, which were washed with five 100-*ul* volumes of wash buffer and eluted with 40 *ul* of 0.1 N NaOH to obtain the non-biotinylated strand. The isolated DNAs were ethanol precipitated and used as templates in a primer extension reaction to begin the next round of selection. Rounds 2-10 were carried out as above, except that the reaction scale was reduced five-fold during the extension step and two-fold during PCR.

The enzymatic DNA molecules thus produced were selected for their ability to cleave a phosphoester that lies within the embedded RNA target sequence. Ten rounds of *in vitro* selective amplification were carried out, based on the enzymatic DNA molecules' activity in the presence of 10 mM Mg<sup>2+</sup> at pH 7.5 and 37°C. During the selection process, there was competition for "preferred" cleavage sites as well as for the "best" catalyst that cleaves at each such preferred site. Two sites and two families of catalysts emerged as possessing the most efficient cleavage capabilities (see Figure 7).

Figure 7 illustrates some of the results of ten rounds of *in vitro* selective amplification carried out essentially as described herein. As shown, two sites and two families of catalysts emerged as displaying the most efficient cleavage of the target sequence. Cleavage conditions were essentially as indicated in Figure 7, namely, 10mM Mg<sup>2+</sup>, pH 7.5, and 37°; data collected after the reaction ran for 2 hours is shown. Cleavage (%) is shown plotted against the number of generations (here, 0 through 10). The number/prevalence of catalytic DNA molecules capable of cleaving the target sequence at the indicated sites in the

substrate is illustrated via the vertical bars, with cleavage at GUUAACUAGAGAU shown by the striped bars, and with cleavage at GUAACUA!GAGAU illustrated via the open (lightly-shaded) bars. In Figure 7, as herein, the arrow (!) indicates the site between two neighboring nucleotides at which cleavage occurs.

Various individuals from the population obtained after the 8th and 10th rounds of selective amplification were cloned. The nucleotide sequences of 29 individuals from the 8th round and 32 individuals from the 10th round were then determined (see Tables 2 and 3, respectively).

Under the heading "Nucleotide Sequence" in each of Tables 2 and 3 is shown the portion of each identified clone that corresponds to the 50 nucleotides that were randomized in the starting pool (i.e., N<sub>50</sub>); thus, the entire nucleotide sequence of a given clone generally includes the nucleotide sequences preceding, following, and including the "N<sub>50</sub>" segment, presuming the substrate sequence is attached and that self-cleavage has not occurred. For example, the entire sequence of a (non-self-cleaved) clone may generally comprise residue nos. 1-33 of SEQ ID NO 50, followed by the residues representing the randomized N<sub>50</sub> region, followed by residue nos. 84-98 of SEQ ID NO 50, or by residue nos. 1-34 of SEQ ID NO 51, followed by the residues representing the randomized N<sub>50</sub> region, followed by residue nos. 85-99 of SEQ ID NO 51. It is believed, however, that the N<sub>50</sub> (or N<sub>40</sub>) region -- or a portion thereof -- of each clone is particularly important in determining the specificity and/or activity of a particular enzymatic DNA molecule. This is particularly evident in reactions in which the substrate and the DNAzyme are separate molecules (see, e.g., Figs. 8 and 9).

Clone numbers are designated as 8-x or 10-x for

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individuals obtained after the 8th or 10th rounds,  
respectively. SEQ ID NOS are also listed and correspond to  
the "N<sub>50</sub>" region of each clone.

5

**Table 2**  
**Cloned Individuals from 8th Round of Amplification**

	<u>Clone No.</u>	<u>SEQ ID NO</u>	<u>"N<sub>50</sub>" Nucleotide Sequence (5'-3')</u>
10	8-2	52	CCA ATA GTG CTA CTG TGT ATC TCA ATG CTG GAA ACA CGG GTT ATC TCC CG
	8-4	53	CCA AAA CAG TGG AGC ATT ATA TCT ACT CCA CAA AGA CCA CTT TTC TCC CG
	8-5 <sup>1</sup>	54	ATC CGT ACT AGC ATG CAG ACA GTC TGT CTG CTT TTT CAT TAC TCA CTC CC
15	8-14	55	CAA TTC ATG ATG ACC AAC TCT GTC AAC ACG CGA ACT TTT AAC ACT GGC A
	8-17 <sup>2</sup>	56	CTT CCA CCT TCC GAG CCG GAC GAA GTT ACT TTT TAT CAC ACT ACG TAT TG
	8-3	57	GGC AAG AGA TGG CAT ATA TTC AGG TAA CTG TGG AGA TAC CCT GTC TGC CA
20	8-6	58	CTA GAC CAT TCA CGT TTA CCA AGC TAT GGT AAG AAC TAG AAT CAC GCG TA
	8-8	59	CGT ACA CGT GGA AAA GCT ATA AGT CAA GTT CTC ATC ATG TAC CTG ACC GC
	8-10	60	CAG TGA TAC ATG AGT GCA CCG CTA CGA CTA AGT CTG TAA CTT ATT CTA CC
25	8-22	61	ACC GAA TTA AAC TAC CGA ATA GTG TGG TTT CTA TGC TTC TTC CCT GA
	8-11	62	CAG GTA GAT ATA ATG CGT CAC CGT GCT TAC ACT CGT TTT ATT AGT ATG TC
	8-21	63	CCC TAC AAC ACC ACT GGG CCC AAT TAG ATT AAC GCT ATT TTA TAA CTC G
30	8-12	64	CCA AAC GGT TAT AAG ACT GAA AAC TCA ATC

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			AAT AGC CCA ATC CTC GCC C
	8-13	65	CAC ATG TAT ACC TAA GAA ATT GGT CCC GTA
			GAC GTC ACA GAC TTA CGC CA
5	8-23	66	CAC AAC GAA AAC AAT CTT CCT TGG CAT ACT
			GGG GAG AAA GTC TGT TGT CC
	8-40	67	CAC ACG AAC ATG TCC ATT AAA TGG CAT TCC
			GTT TTT CGT TCT ACA TAT GC
	8-24	68	CAG AAC GAG GGT CTT GTA AGA CTA CAC CTC
			CTC AGT GAC AAT AAT CCT G
10	8-26	69	CAC TAC AGC CTG ATA TAT ATG AAG AAC AGG
			CAA CAA GCT TAT GCA CTG G
	8-27	70	GGG TAC ATT TAT GAT TCT CTT ATA AAG AGA
			ATA TCG TAC TCT TTT CCC CA
	8-28	71	CCA AAG TAC ATT CCA ACC CCT TAT ACG TGA
15			AAC TTC CAG TAG TTT CCT A
	8-29	72	CTT GAA GAT CCT CAT AAG ACG ATT AAA CAA
			TCC ACT GGA TAT AAT CCG GA
	8-34	73	CGA ATA GTG TCC ATG ATT ACA CCA ATA ACT
			GCC TGC CTA TCA TGT TTA TG
20	8-35	74	CCA AGA GAG TAT CGG ATA CAC TTG GAA CAT
			AGC TAA CTC GAA CTG TAC CA
	8-36	75	CCA CTG ATA AAT AGG TAA CTG TCT CAT ATC
			TGC CAA TCA TAT GCC GTA
	8-37	76	CCC AAA TTA TAA ACA ATT TAA CAC AAG CAA
25			AAG GAG GTT CAT TGC TCC GC
	8-39	77	CAA TAA ACT GGT GCT AAA CCT AAT ACC TTG
			TAT CCA AGT TAT CCT CCC CC

30           <sup>1</sup> identical to 10-4, 10-40

<sup>2</sup> identical to 8-20, 8-32, 8-38, 10-1, 10-34; 1  
mutation to 10-11; 3 mutations to 10-29

## Cloned Individuals from 10th Round of Amplification

	<u>Clone No.</u>	<u>SEQ ID NO</u>	<u>"N<sub>50</sub>" Nucleotide Sequence (5'→3')</u>
5	10-3 <sup>3</sup>	78	CCG AAT GAC ATC CGT AGT GGA ACC TTG CTT TTG ACA CTA AGA AGC TAC AC
	10-10	79	CCA TAA CAA ATA CCA TAG TAA AGA TCT GCA TTA TAT TAT ATC GGT CCA CC
	10-12	80	CAG AAC AAA GAT CAG TAG CTA AAC ATA TGG TAC AAA CAT ACC ATC TCG CA
10	10-14	81	CCT TTA GTT AGG CTA GCT ACA ACG ATT TTT CCC TGC TTG GCA ACG ACA C
	10-15	82	CTC CCT ACG TTA CAC CAG CGG TAC GAA TTT TCC ACG AGA GGT AAT CCG CA
	10-19	83	CGG CAC CTC TAG TTA GAC ACT CCG GAA TTT TTC CCC
15	10-39	84	CGG CAC CTC TAG TTA GAC ACT CCG GAA TTT TAG CCT ACC ATA GTC CGG T
	10-23	85	CCC TTT GGT TAG GCT AGC TAC AAC GAT TTT TCC CTG CTT GAA TTG TA
	10-27 <sup>4</sup>	86	CCC TTT GGT TAG GCT AGC TAC AAC GAT TTT TCC CTG CTT GAC CTG TTA CGA
20	10-31	87	CCT TTA GTT AGG CTA GCT ACA ACG ATT TTT CCC TGC TTG GAA CGA CAC
	10-18	88	CAT GGC TTA ATC ATC CTC AAT AGA AGA CTA CAA GTC GAA TAT GTC CCC CC
	10-20	89	CAA CAG AGC GAG TAT CAC CCC CTG TCA ATA GTC GTA TGA AAC ATT GGG CC
25	10-6	90	TAC CGA CAA GGG GAA TTA AAA GCT AGC TGG TTA TGC AAC CCT TTT CGC A
	10-7	91	CTC GAA ACA GTG ATA TTC TGA ACA AAC GGG TAC TAC GTG TTC AGC CCC C
	10-8	92	CCA ATA ACG TAA CCC GGT TAG ATA AGC ACT TAG CTA AGA TGT TTA TCC TG

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	10-16	93	CAA TAC AAT CGG TAC GAA TCC AGA AAC ATA ACG TTG TTT CAG AAT GGT CC
	10-21	94	GCA ACA ACA AGA ACC AAG TTA CAT ACA CGT TCA TCT ATA CTG AAC CCC CA
5	10-24	95	CCT TTG AGT TCC TAA ATG CCG CAC GGT AAG CTT GGC ACA CTT TGA CTG TA
	10-28	96	CAA AGA TCT CAC TTT GGA AAT GCG AAA TAT GTA TAT TCG CCC TGT CTG C
	10-33	97	CCA CGT AGA ATT ATC TGA TTT ATA ACA TAA CGC AGG ATA ACT CTC GCC CA
10	10-35	98	CAC AAG AAA GTG TCG TCT CCA GAT ATT TGA GTA CAA GGA ACT ACG CCC
	10-36	99	CAT GAA GAA ATA GGA CAT TCT ACA GGC TGG ACC GTT ACT ATG CCT GTA GG
15	10-37	100	CAT AGG ATA ATC ATG GCG ATG CTT ATG ACG TGT ACA TCT ATA CCT T
	10-38	101	CAG ATG ATC TTC CTT TAA AGA CTA CCC TTT AAA GAA ACA TAA GGT ACC CC

20                   <sup>3</sup> 1 mutation to 10-5

<sup>4</sup> 1 mutation to 10-30

25                 The self-cleavage activity of various clones was  
               subsequently measured. Clones 8-5, 8-17, and 10-3 were  
               found to cleave efficiently at the site 5' GUAACU!AGAGAU 3',  
               while clones 10-14, 10-19 and 10-27 were found to cleave  
               efficiently at the site 5' G!UAACUAGAGAU 3'. When the RNA  
               portion of the molecule was extended to the sequence 5'  
30                 GGAAAAAGUAACUAGAGAUGGAAG 3' (residue nos. 1-24 of SEQ ID NO  
               51), clones 8-17, 10-14, and 10-27 retained full activity,  
               while clones 8-5, 10-3, and 10-19 showed diminished  
               activity. Subsequently, clone 10-23 was found to exhibit a  
               high level of activity in the self-cleavage reaction

involving the extended RNA domain.

It should also be noted, in the event one of skill in the relevant art does not appreciate same, that the nucleotide sequences preceding and following the "N<sub>50</sub>" segments of the polynucleotide molecules engineered according to the teachings of the present invention disclosure, i.e., the substrate binding regions flanking the "N<sub>50</sub>" region, may be altered in a variety of ways in order to generate enzymatic DNA molecules of particular specificities, such as by length, nucleotide sequence, type of nucleic acid, and the like. For example, while residue nos. 1-24 of SEQ ID NO 51 are described herein as RNA nucleotides, they may alternatively comprise DNA, RNA, or composites thereof. (Thus, for example, SEQ ID NO 51 could easily be altered so that nucleic acid residue nos. 1-7 would comprise DNA, residue nos. 8-19 would comprise RNA, residue nos. 20-99 would comprise DNA, and so on.) Similarly, the nucleotides following the "N<sub>50</sub>" region may comprise RNA, DNA, or composites thereof. The length of the regions preceding and following the "N<sub>50</sub>" (or "N<sub>40</sub>" -- see Example 4) region(s) may also be varied, as disclosed herein. Further, sequences preceding and/or following N<sub>50</sub> or N<sub>40</sub> regions may be shortened, expanded, or deleted in their entirety.

Moreover, as noted above, we selected a specific region of HIV-1 RNA as the target sequence in the methods described in this Example; such a sequence is not the only sequence one may use as a target. Clearly, one of skill in the relevant art may follow our teachings herein to engineer and design enzymatic DNA molecules with specificity for other target sequences. As disclosed herein, such target sequences may be constructed or inserted into larger sequences comprising DNA, RNA, or composites thereof, as

illustrated by SEQ ID NOS 50 and 51.

The self-cleavage reaction was easily converted to an intermolecular cleavage reaction by dividing the enzyme and substrate domains into separate molecules. Clones 8-17 and 10-23 were chosen as prototype molecules. Both were shown to act as DNA enzymes in the cleavage of a separate all-RNA substrate in a reaction that proceeds with multiple turnover (Figure 8). The substrate binding arms were subsequently reduced to 7 base-pairs on each side of the unpaired nucleotide that demarcates the cleavage site (Figure 9).

Figure 8 illustrates the nucleotide sequences, cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 and 10-23. Reaction conditions were as shown, namely, 10mM Mg<sup>2+</sup>, pH 7.5, and 37°C. The DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3') -- which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 is shown on the right, with the site of cleavage of the RNA substrate indicated by the arrow. Again, the substrate sequence is indicated. For the 8-17 enzyme, the turnover rate was approximately 0.6 hr<sup>-1</sup>; for the 10-23 enzyme, the turnover rate was approximately 1 hr<sup>-1</sup>.

As illustrated in Figure 8, the nucleotide sequence of the clone 8-17 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows:

5'-CTTCCACCTTCCGAGCCGGACGAAGTTACTTTT-3' (residue nos. 1-34 of SEQ ID NO 56). In that same figure, the nucleotide sequence of the clone 10-23 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows:  
5'-CTTGTTAGGCTAGCTACAACGATTTTCC-3' (residue nos. 3-33 of

SEQ ID NO 85).

Figure 9 further illustrates the nucleotide sequences, cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 and 10-23.

5 Reaction conditions were as shown, namely, 10mM Mg<sup>2+</sup>, pH 7.5, and 37°C. As in Figure 8, the DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3') --

10 which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 is shown on the right, with the site of cleavage of the RNA substrate indicated by the arrow. Again, the substrate sequence is indicated. For

15 the 8-17 enzyme,  $k_{obs}$  was approximately 0.002 min<sup>-1</sup>; for the 10-23 enzyme, the value of  $k_{obs}$  was approximately 0.01 min<sup>-1</sup>.

As illustrated in Figure 9, the nucleotide sequence of the clone 8-17 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows:

20 5'-CCACCTTCCGAGCCGGACGAAGTTACT-3' (residue nos. 4-30 of SEQ ID NO 56). In that same figure, the nucleotide sequence of the clone 10-23 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows:

25 5'-CTAGTTAGGCTAGCTACAAACGATTTTCC-3' (residue nos. 5-33 of SEQ ID NO 85, with "CTA" substituted for "TTG" at the 5' end).

The catalytic rate of the RNA-cleaving DNA enzymes has yet to be fully optimized. As disclosed above and as reported in previous studies, we have been able to improve

30 the catalytic rate by partially randomizing the prototype molecule and carrying out additional rounds of selective amplification. We have found, however, that the  $K_m$  for Mg<sup>2+</sup> is approximately 5 mM and 2 mM for the 8-17 and 10-23 DNA

enzymes, respectively, measured at pH 7.5 and 37°C; this is certainly compatible with intracellular conditions.

6. Preparation of a Universal Substrate Enzyme

5       The foregoing has shown that an enzyme of the present invention has the ability to catalytically cleave target nucleic acids having preselected sequences. Furthermore, an enzyme is shown to cleave a nucleic acid which is all DNA, or which has a ribonucleotide (i.e., an RNA component) imbedded within a deoxyribonucleotide (i.e., DNA) sequence, or which is entirely DNA. In addition, it is seen that the substrate can be altered and an enzyme is prepared which can cleave that substrate.

10      By the present invention, an enzyme is provided that can cleave any targeted substrate, i.e., one containing a preselected nucleotide sequence in the length range of about 10-30 nucleotides, in a kinetic manner that is efficient and specific under physiological conditions. This enzyme is easy to manufacture and inexpensive, and is useful for 15     inactivation of target RNA, particularly messenger RNA (mRNA), for probing the structure of RNA and for the manipulation of recombinant RNA, such as a sequence-specific endoribonuclease.

20      For inactivation of RNAs, the enzyme is particularly useful for inactivation of target cellular RNAs, such as an "antisense" oligodeoxynucleotide can be used, to block mRNA function. However, the present enzyme is superior to an antisense reagent because the present enzyme provides both target recognition and cleavage, and can function with 25     catalytic turnover, whereas an antisense molecule only provides recognition in a equimolar manner (i.e. non-catalytic), and must rely upon other cellular enzymes for 30     the RNA inactivation reactions.

The following section describes the preparation of improved enzymes based on the "10-23" and the "8-17" motifs described above. These improved enzymes are generic enzymes which can cleave any preselected target sequence, and that  
5 target specificity depends solely on the sequence of the substrate binding regions of the enzyme, as described further herein.

As described in Example 5, above, two motifs, designate "10-23" and "8-17", were identified during sequential rounds  
10 of selective amplification and recast into an intermolecular cleavage reaction, and shown to perform efficiently in this manner.

Further studies were conducted using a reaction that yielded site-specific catalytic cleavage of the separate  
15 substrate molecule shown in Figure 9 (i.e., intermolecular cleavage) under simulated physiological conditions of 2 mM MgCl<sub>2</sub>, 150 mM KCl, pH 7.5, 37 C, for a rate of about  $k_{cat} = 0.01 \text{ min}^{-1}$ .

Cleavage occurred following an unpaired purine  
20 nucleotide of the substrate that was flanked by oligonucleotides complementary to the enzyme. The 5' and 3' cleavage products bore a 2' (3') phosphate and 5' hydroxyl, respectively, indicative of a reaction mechanism involving attack by a 2' hydroxyl on an adjacent phosphate. For both  
25 the 8-17 and 10-23 motif enzymes, the sequence of the substrate can be changed without loss of catalytic activity, so long as the substrate-binding arms of the enzyme were changed in a complementary manner. The 8-17 enzyme had a special requirement for a rG-dT "wobble" pair located  
30 immediately downstream from the cleavage site. Substitution of a Watson-Crick pair at this position eliminated catalytic activity. The substrate-binding arms of the 10-23 enzyme interacted with the substrate entirely through standard

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Watson-Crick pairing. The catalytic core of the 8-17 and 10-23 motif enzymes, located between the two substrate-binding arms, contained 13 and 15 deoxynucleotides, respectively.

5 In order to define more precisely the sequence requirements of the catalytic core, a library of  $10^{14}$  variants of each motif was generated, introducing random mutations at a frequency of 25% per nucleotide position throughout the core. Each library was subjected to six  
10 different in vitro selection protocols, involving a total of 52 rounds of selective amplification. The method and stringency of selection were varied in order to conduct a thorough examination of sequences related to the two prototype molecules. Individuals from the selected  
15 populations were cloned, sequenced, and tested for catalytic activity. This procedure was carried out as follows.

The re-selections based on the 8-17 and 10-23 molecules involved six different lineages for each motif. Each lineage entailed 5-21 rounds of in vitro selection, differing with respect to the selection protocol and reaction times. All cleavage reactions were carried out in 2 mM MgCl<sub>2</sub>, 150 mM NaCl, and 50 mM Tris\*HCl (pH 7.5) at 37 C. Reaction times varied from 60 min in early rounds to 1 min in later rounds. Each starting pool of templates was based on a sequence complementary to the prototype, with fixed binding arms of seven nucleotides each and a catalytic core randomized to 25% degeneracy at each nucleotide position. For the 8-17 and 10-23 motifs, the templates had the sequence  
25 5'-gtgccaagcttaccgagtaactTCG-TCCGGCTCGGRagatgggtcgctgtccttc  
30 cATCTCTAGTTACTTTTC- 3' and  
5'-gttgccaagcttaccg-gaaaaaaTCGTTGTAGCTAGCCtaactaggcgctgtcc  
ttccATCTCTAGT TACTTTTC-3', respectively (PCR primer sites

in lower case; substrate-binding arms underlined; randomized positions italicized). The primer used in the template-directed extensions had the sequence 5'-biotin-r(GGAAAAAA-GUAACUAGAGAUGG)d(AAGAGATGGCGAC)-3'. The 5 PCR primers for the 8-17-based selections were 5'-GTGCCAAGCTTACCGAGTAACT-3' and 5'-d(GGAAGGACAGACGACC-CATC)rU and for the 10-23-based selections were 5'-GTGCCAAGCTTACCGGGAAAAA-3' and 5'-d(GGAAGGACAGACGACCTAGTT)rA. The PCR primers encompassed 10 the binding arms, thus fixing these sequences. One of the PCR primers in each set contained a 3'-terminal ribonucleotide, allowing isolation of the template strand from the double-stranded PCR products by alkaline hydrolysis 15 of the non-template strand and subsequent purification by polyacrylamide gel electrophoresis. A gel-based selection scheme was employed in some of the lineages. In those cases, the PCR primers were 5'-biotin-GTGCCAAGCTTACCG-3' and 5'-GAAAAAGTAACTAG-AGATGGAAGGACAGACGACC-3' and the extension reactions were carried out on the solid support using the 20 primer 5'-r(GGAAAAGUAACUAGAGAUGGAAG)-3'. A trace amount of [ $\alpha$ -32P]-dATP was included in the mixture to label the extension products, which were eluted with alkali, purified by denaturing polyacrylamide gel purification, and recovered 25 by electroelution. The molecules then were reacted and those that underwent cleavage were isolated by gel electrophoresis.

Following the eighth and tenth rounds of the initial selection, individual molecules were cloned and sequenced, as described above. The 17th clone from round eight (8-17) 30 and 23rd clone from round ten (10-23) had the sequence 5'-cacggttcga-atggcGTTATGCATCACACTATTTTCATTGAAGCAGGCCGAGCCT T CCACCTTCcagcgtag-agaagg-3' and 5'-cacggttcgaatggcATGTTAAGTTCGTCCCTTTAGCAACATCGATCGGATT-GG

TTTCCCCcagcggtagagaagg-3', respectively (primer sites in lower case; substrate-binding arms underlined). For the intermolecular reaction, synthetic oligodeoxynucleotides were prepared based on the cloned sequences but lacking the regions outside of the substrate-binding arms. These were used to cleave an all-RNA substrate having the same sequence as the primer used to construct the initial library (see above). Subsequently, the substrate-binding arms of the DNA enzyme were reduced to seven nucleotides each and made perfectly complementary to the RNA substrate.

In the case of the 8-17 enzyme, sequence variation among the cloned individuals suggested that the catalytic core consisted of a short internal stem-loop followed by an unpaired region of 4-5 nucleotides (Figure 10). The stem always contained three base-pairs, at least two of which were G-C. The loop was invariant, having the sequence 5'-AGC-3'. Synthetic constructs in which the stem was lengthened or the sequence of the loop was altered did not exhibit catalytic activity. The unpaired region, connecting the 3' half of the stem to the downstream substrate-binding domain, had the sequence 5'-WGR-3' or 5'-WCAA-3' (W = A or T; R = A or G). Variants having the sequence 5'-TCGAA-3' in this region exhibited the highest level of catalytic activity, but this enhancement relative to the 8-17 enzyme was not generalizable to other substrate sequences.

The eighth nucleotide position of the catalytic core of the 10-23 enzyme motif allows variation as either T, C, or A, although a T at this position (as in the prototype) provided the highest level of activity. A survey of numerous different combinations of RNA substrate and corresponding complementary DNA enzyme in the substrate binding region revealed that the 10-23 motif was generalizable with respect to any substrate sequence.

Catalytic activities for the 10-23 and 8-17 motif variants were then measured using multiple-turnover reactions, typically exhibiting < 20% variation for identical experiments performed on different days. Kinetic values obtained in single- and multiple-turnover experiments were similar; values obtained with synthetic RNA substrates were slightly less favorable than those obtained with *in vitro* transcribed substrates. Reported  $k_{cat}$  and  $K_m$  values were determined from the  $y$ -intercept and negative slope, respectively, of the best-fit line to a modified Eadie-Hofstee plot of  $k_{obs}$  vs.  $k_{obs} / [S]$ . Each plot consisted of ten data points for a range of  $[S]$  that spanned  $K_m$ , with  $[S]$  in a 10-fold excess over  $[E]$ .  $k_{obs}$  values were typically based on five data points obtained over the first 10% of the reaction. Substrate and enzyme molecules were preincubated separately for 10 min in reaction buffer, then combined to initiate the reaction. All reactions were carried out in the presence of 0.01% sodium dodecyl sulfate to prevent adherence of material to the vessel walls. The pH was maintained by addition of 50 mM 4-(2-hydroxyethyl)-piperazine-1-propanesulfonic acid. Kinetic values did not depend on the identity of the buffer. Reaction products were separated by electrophoresis in a denaturing 20% polyacrylamide gel and quantitated using a phosphorimager.

Cleavage occurred on the 3' side of a single unpaired nucleotide, preferably a purine, that was followed by a pyrimidine. Target sites surrounded by A and U were cleaved most efficiently, with a catalytic rate of approximately 0.1 min<sup>-1</sup> under simulated physiological conditions.

A DNA enzyme that cleaves RNA at an A\*U site can be used to target any mRNA start codon (A\*UG). As a test case, both synthetic and *in vitro* transcribed versions of a 17mer

RNA corresponding to the translation initiation region of HIV-1 gag/ pol mRNA (5'-GGAGAGAGA\*UGGGUGCG-3') were prepared. Both versions of the substrate were cleaved at the expected position by the corresponding 10-23 DNA enzyme, 5 in a reaction that proceeded with a kcat of 0.15 min-1 and Km of 0.47 nM under simulated physiological conditions (catalytic efficiency, kcat / Km = 3.2 x 108 M-1 min-1) (Figure 11). The catalytic rate increased with increasing MgCl<sub>2</sub> concentration range of 1-250 mM, with an apparent Km 10 for Mg<sup>2+</sup> of 180 mM at pH 7.5 and 37 °C. The catalytic rate increased in a roughly log-linear fashion with increasing pH over the range 7.0-8.5 consistent with a reaction mechanism involving deprotonation of the 2'-hydroxyl that lies adjacent to the cleaved phosphoester. In the presence of 50 mM MgCl<sub>2</sub> at pH 8.0 and 37 °C, conditions that are useful in 15 the laboratory manipulation of RNA, kcat was 3.4 min-1 and Km was 0.76 nM. The catalytic efficiency of the 10-23 DNA enzyme, under both physiological and laboratory conditions, compares favorably with that of known RNA-cleaving RNA 20 enzymes. Compared to the protein enzyme ribonuclease A, the DNA enzyme has ~104-fold lower kcat but ~105-fold more favorable Km.

The 10-23 enzyme can be used to cleave a variety of biologically relevant RNAs. We prepared synthetic RNA 25 substrates corresponding to 15-17 nucleotides surrounding the translation initiation site of HIV-1 gag / pol, env, vpr, tat, nef IGF-R and E100 ligase mRNA. Each was cleaved at the expected position by a synthetic DNA enzyme that contained the 10-23 catalytic core flanked by 30 substrate-binding arms of 7 to 12 nucleotides each (Table 4). In all cases the catalytic rate was about 0.1 min-1 under simulated physiological conditions. The value for Km, however, varied with the nucleotide composition of the

substrate. For the guanosine-rich gag / pol substrate, Km was < 1 nM when either the 7- or 8-nucleotide substrate-binding arms were employed. The env and vpr substrates were cleaved with a much less favorable Km when the 7-nucleotide binding arms were used, but Km improved substantially when the arms were increased to 8 nucleotides each.

TABLE 4

10 DNA-catalyzed cleavage of various RNA substrates under simulated physiological conditions

	<u>Gene</u>	<u>SEQ ID NO</u>	<u>Target Sequence</u>	<u>Arm length</u>	<u>kcat (min-1)</u>	<u>Km (nM)</u>
15	<u>HIV-1</u>					
	gag	102	GGAGAGAGA*UGGGUGCG	8+8	0.1	0.7
	gag	103	GAGAGAGA*UGGGUGC	7+7	0.1	0.9
	env	104	CAGUGGCAA*UGAGAGUG	8+8	0.04	9
	env	105	AGUGGCAA*UGAGAGU	7+7	0.03	900
20	vpr	106	GAGGAUAGA*UGGAACAA	8+8	0.1	20
	vpr	107	AGGAUAGA*UGGAACA	7+7	0.08	500
	tat	108	GCAAGAAA*UGGAGCC	7+7	0.04	300
	nef	109	CUAUAAAGA*UGGGUGA	7+7	0.05	900
25	<u>FIV</u>					
	gag	110	UACAGCAACA*UGGGGAAUGG	9+10	0.005	8
	gag	111	CAUGGGGAA*UGGACAGGG	8+9	0.006	5
<u>IGF-R</u>						
30		112	CAAAUAAAAGGGA*UGAAGUCUGG	12+10	0.02	20
		113	AAGGAAUGAAG*UCUGGCUCCG	10+10	0.3	50
		114	AUACCGCAAAG*UCUUUGAGAAUU	10+12	0.1	30
		115	AAGUCUUUGAGAG*UUUCCUGCAC	12+10	0.05	21
		116	AACACCACCA*UGUCCAGCC	9+9	0.06	2

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117	GGCCUUUCACA*UUGUACCGC	10+9	0.1	10
118	UUGUACCGCA*UCGAUAUCCAC	9+11	0.06	1
E100 ligase				
	119 GAACAUUACAUUA*UAGUGACCAG	12+10	1.0	80

5

Kinetic values measured and shown in Table 4 were obtained under multiple-turnover conditions, with synthetic RNA substrate in >10-fold excess over synthetic DNA enzyme, using the reaction conditions: 2 mM MgCl<sub>2</sub>, 150 mM NaCl, pH 7.5, 37 C, obtained at 25 mM MgCl<sub>2</sub>.

As a further demonstration of the variation to the substrate binding region (arms) that a DNA enzyme of the present invention can support, the length of the arms was systematically varied from 4 to 13 nucleotide residues using the HIV-1 gag gene start codon target nucleotide sequence shown in Table 4. The reaction was run separately for each DNA enzyme construct prepared as described above except that the arm length was varied as shown in Figure 13. The kinetic properties of the catalytic DNA enzyme was measured as before under the conditions of 2 mM Mg<sup>2+</sup>, 150 mM NaCl, pH 7.5, 37 C and measuring multiple turnover of enzyme. Both  $k_{cat}$  (min<sup>-1</sup>) and  $K_m$  (nM) was measured, and is shown in Figure 13 using the 10-23 motif modified for complementary binding to the HIV-1 gag gene.

The results show that useful catalytic rates are observed over the full range of from 5 to 13 nucleotides per arm per substrate binding region, with a preferred range of from 7 to 10 nucleotides being particularly efficient. The results also show that the effective concentration of enzyme for half maximal catalysis ( $K_m$ ) is in the range of 1000 to 0.05 nanomolar (nM) for arms of a 5 to 13 nucleotide length, with lengths of 7 to 13 particularly preferred.

A variety of modifications were incorporated into the 10-23 DNA enzyme and tested for stability in 10% fetal bovine serum (Figure 13). These modifications included: 1) an "inverted" (3',3'-linked) thymidylate at the 3' end of the DNA; 2) five 2'-O-methyl residues at the distal end of both substrate-binding arms; 3) 2'-O-methyl residues at all positions in both substrate-binding arms; 4) phosphorothioate residues at the five pyrimidine-pyrimidine sites within the catalytic core; 5) three phosphorothioate residues at the distal end of both substrate-binding arms. The best protection was afforded by the inverted thymidylate ( $t_{1/2} > 60$  min). All of the other modifications resulted in enhanced serum stability compared to the unmodified DNA, with the exception of the five P=S substitutions in the core. All of the modified DNA enzymes retained catalytic activity.

Thus, the invention describes a catalytic DNA molecule having site-specific endonuclease activity which can be designed to cleave any preselected substrate nucleic acid sequence. The DNA molecule (enzyme) has first and second substrate binding regions (arms) flanking a core region, and each arm has a sequence complementary to a portion of the target substrate nucleic acid sequence, such that together, the first and second arms define the substrate nucleic acid sequence to be cleaved. By complementary is meant that the substrate binding regions bind to the target sequence using conventional Watson-Crick base pairing.

The arms can be any of a variety of lengths. However, because it is seen that the length affects the catalytic rate ( $k_{cat}$ ) and effective concentration ( $K_m$ ) of the enzyme (see Figure 12), the preferred arm lengths are from 5 to 13 nucleotides of complementarity each, preferably about 6 to 11 nucleotides, and more preferably about 7 to 10

nucleotides in length.

The core region can have a variety of sequences based on the formula:

(I.) T(stem)'AGC(stem)"Z,

5 wherein said (stem)' and (stem)" are each three sequential nucleotides which when hybridized as a (stem)':(stem)" pair comprise three base pairs including at least two G:C pairs and wherein said Z = WCGR or WCGAA, and W = A or T and R = A or G. This stem structure is shown in Figure 10 and  
10 illustrates three complementary nucleotide pairs.

Particularly preferred is the prototype structure of the core region of motif 8-17, "TCCGAGCCGGACGA" (SEQ ID NO 120) shown in Figure 10.

In another embodiment, the core region can have a  
15 sequence according to the formula:

(II.) RGGCTAGCXACAACGA (SEQ ID NO 122),

wherein said X = T, C or A, and R = A or G. Particularly preferred is the prototype structure of the core region of motif 10-23, "RGGCTAGCTACAACGA" (SEQ ID NO 121) shown in  
20 Figure 10.

A DNA enzyme of the above design can exhibit a range of useful catalytic rates and effective catalytic concentrations depending upon the arm length as noted in Figure 12. A DNA enzyme of this invention according to the 25 8-17 or 10-23 motif typically has a catalytic turnover rate ( $k_{cat}$ ) of about 0.005 to 0.1 min<sup>-1</sup>, preferably about 0.01 to 0.1 min<sup>-1</sup>, and more preferably about 0.03 to 0.1 min<sup>-1</sup>, under physiological conditions. A particularly preferred enzyme has a rate of about 0.1 min<sup>-1</sup>. A DNA enzyme of this 30 invention according to the 8-17 or 10-23 motif typically also has a catalytic half maximal effective concentration ( $K_m$ ) of about 0.05 to 1000 nanomolar (nM), preferably about 0.7 to 900 nM, more preferably less than about 1.0

micromolar ( $\mu\text{M}$ ), and most preferably about 0.1 ( $\mu\text{M}$ ), under physiological conditions.

The foregoing also demonstrates that a preferred DNA enzyme has nucleotide modifications which stabilize the DNA enzyme for use under physiological conditions. Any of a variety of modifications can be utilized so long as the Enzyme retains its catalytic activity as defined and measured herein, and therefore the invention need not be so limited to a particular stabilization modification.

Preferred modifications render a subject DNA molecule less susceptible to exo or endo nucleolytic digestion. In one embodiment the modification comprises incorporation of one or more nucleoside phosphorothioate residues, such as is described by Zhang et al, Biochem. Pharmacol., 50:545-556 (1995) or by Stein, C.A., Trends Biotechnol., 14:147-149 (1996). The phosphorothioate residue can be in the arms or in the core, and in one embodiment can comprise a residue on a dipyrimidine within the core. Another modification comprises the substitution of an O-methyl group onto the 2' position of the ribose or deoxyribose component of the sugar nucleotide, to form a 2' O-methyl ribonucleotide, as well known and described by Zhang et al., above. An additional modification is to attach an inverted terminal nucleotide onto the 3' end of the DNA molecule, such that the 3' end is blocked, appearing as a free 5' end. This structure blocks 3'endonucleases, and is produced by forming a 3'-3' linked nucleotide at the 3' end (i.e, an inverted nucleotide). The preparation of a 3' inverted nucleotide at the 3' end is well known, and is prepared using a modified solid support in oligonucleotide synthesis having the terminal 3' residue inverted as a starting material. A preferred solid support material for this purpose is dT-5'-CPG 500, available from Glen Research (Sterling, VA) and is a modified controlled

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pore glass resin having the modified residue.

The foregoing specification, including the specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (I) APPLICANT: The Scripps Research Institute  
5 (ii) TITLE OF INVENTION: ENZYMATIC DNA MOLECULES  
(iii) NUMBER OF SEQUENCES: 101  
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10 (C) CITY: La Jolla  
(D) STATE: California  
(E) COUNTRY: United States  
(F) ZIP: 92037

## 15 (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

20 (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER: PCT/US95/

- (B) FILING DATE: 01-DEC-1995  
(C) CLASSIFICATION:

25 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: US 08/472,194

- (B) FILING DATE: 07-JUN-1995

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(A) APPLICATION NUMBER: US 08/349,023  
(B) FILING DATE: 02-DEC-1994

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(C) REFERENCE/DOCKET NUMBER: 463.2 PC

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- 40 (A) TELEPHONE: (619) 554-2937

-100-

(B) TELEFAX: (619) 554-6312

(2) INFORMATION FOR SEQ ID NO:1:

5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15

CGGTAAGCTT GGCAC

15

(2) INFORMATION FOR SEQ ID NO:2:

20

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

30

- (A) NAME/KEY: misc\_difference
- (B) LOCATION: replace(8, "")
- (D) OTHER INFORMATION: /standard\_name= "ADENOSINE  
RIBONUCLEOTIDE"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCACTATNAG GAAGAGATGG

20

5 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- 10 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACACATCTCT GAAGTAGCGC CGCCGTATAAG TGACGCTA

38

20 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 80 base pairs
- (B) TYPE: nucleic acid
- 25 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGCCAAGCT TACCGNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN 60  
NNNNNGTCGC CATCTTTCC 80

35

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## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

10

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 28
- (D) OTHER INFORMATION: /standard\_name= "2'3' CYCLIC PHOSPHATE"

## (ix) FEATURE:

- (A) NAME/KEY: misc\_difference
- (B) LOCATION: replace(28, "")
- (D) OTHER INFORMATION: /standard\_name= "ADENOSINE RIBONUCLEOTIDE"

## 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGACGAATT CTAATACGAC TCACTATN

28

## (2) INFORMATION FOR SEQ ID NO:6:

30

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

40

## (ix) FEATURE:

-103-

- (A) NAME/KEY: misc\_difference
- (B) LOCATION: replace(28, "")
- (D) OTHER INFORMATION: /standard\_name= "ADENOSINE RIBONUCLEOTIDE"

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGACGAATT CTAATACGAC TCACTATN

28

10 (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20 (ix) FEATURE:

- (A) NAME/KEY: misc\_difference
- (B) LOCATION: replace(8, "")
- (D) OTHER INFORMATION: /standard\_name= "ADENOSINE RIBONUCLEOTIDE"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCACTATNGG AAGAGATGG

19

30 (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 8 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

40 (ix) FEATURE:

-104-

- (A) NAME/KEY: misc\_difference  
(B) LOCATION: replace(8, "")  
(D) OTHER INFORMATION: /standard\_name= "ADENOSINE  
NUCLEOTIDE"

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCACTATN

8

10 (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
15 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCATCTCTTC CTATAGTGAG TCCGGCTGCA

30

(2) INFORMATION FOR SEQ ID NO:10:

25

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
30 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35

GTGCCAAGCT TACCG

15

(2) INFORMATION FOR SEQ ID NO:10:

40

- (i) SEQUENCE CHARACTERISTICS:

-105-

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

10 CTGCAGAATT CTAATACGAC TCACTATAGG AAGAGATGGC GAC 43

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- 15
- (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- 25
- (A) NAME/KEY: misc\_difference
  - (B) LOCATION: replace(8, "")
  - (D) OTHER INFORMATION: /standard\_name= "ADENOSINE RIBONUCLEOTIDE"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

30 TCACTATNGG AAGAGATGG 19

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- 35
- (A) LENGTH: 43 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (genomic)

-106-

## (ix) FEATURE:

- (A) NAME/KEY: misc\_difference
- (B) LOCATION: replace(28, "")
- (D) OTHER INFORMATION: /standard\_name= "ADENOSINE  
RIBONUCLEOTIDE"

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGGACGAATT CTAATACGAC TCACTATNGG AAGAGATGGC GAC

43

10

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

## (ii) MOLECULE TYPE: DNA (genomic)

20

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCACACATCT CTGAAGTAGC GCCGCCGTAT GTGACGCTAG GGGTTCGCCT

50

25

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

## (ii) MOLECULE TYPE: DNA (genomic)

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGGGGAAACG CCGTAACAAG CTCTGAACTA GCGGTTGCGA TATAGTCGTA

50

## (2) INFORMATION FOR SEQ ID NO:16:

40

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## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

10

CGGGACTCCG TAGCCCATTG CTTTTGCAG CGTCAACGAA TAGCGTATTA

50

(2) INFORMATION FOR SEQ ID NO:17:

15

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCACCATGTC TTCTCGAGCC GAACCGATAG TTACGTCATA CCTCCCGTAT

50

25

(2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA (genomic)

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCCAGATTGC TGCTACCAGC GGTACGAAAT AGTGAAGTGT TCGTGAAT

50

40

(2) INFORMATION FOR SEQ ID NO:19:

-108-

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

10

ATAGGCCATG CTTGGCTAG CGGCACCGTA TAGTGTACCT GCCCTTATCG

50

(2) INFORMATION FOR SEQ ID NO:20:

15

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

25

TCTGCTCTCC TCTATTCTAG CAGTGCAGCG AAATATGTCG AATAGTCGGT

50

(2) INFORMATION FOR SEQ ID NO:21:

30

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

40

TTGCCAGCA TAGTCGGCAG ACGTGGTGT AGCGACACGA TAGGCCGGT

50

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## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTGCTAGCTC GGCTGAAC TT CTGTAGCGCA ACCGAAATAG TGAGGCTTGA

50

## 15 (2) INFORMATION FOR SEQ ID NO:23:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

25 (ix) FEATURE:

- (A) NAME/KEY: misc\_difference
- (B) LOCATION: replace(28, "")
- (D) OTHER INFORMATION: /standard\_name= "ADENOSINE  
RIBONUCLEOTIDE"

30 /label= rA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGGACGAATT CTAATACGAC TCACTATNGG AAGAGATGGC GACATCTCNN NNNNNNNNNN 60

35 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNGT GACGGTAAGC TTGGCAC 107

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 49 base pairs

-110-

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCGGCCCACCT CTTTTACGAG CCTGTACGAA ATAGTGCTCT TGTTAGTAT

49

10

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 48 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTCTTCAGC GATGCACGCT TGTGTTAACATG TTGCACCCAT GTTAGTGA

48

25

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 46 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TCTCATCAGC GATTGAACCA CTTGGTGGAC AGACCCATGT TAGTGA

46

(2) INFORMATION FOR SEQ ID NO:27:

40

-111-

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

10

CCGGCCCACCT CTTTTACGAG CCTGTACGAA ATAGTGTCT TGTTAGTAT

49

(2) INFORMATION FOR SEQ ID NO:28:

15

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

25

CCGGCCCACCT CTTTTACGAG CCTGTACGAA ATAGTGCTCT CGTTAGTAT

49

(2) INFORMATION FOR SEQ ID NO:29:

30

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

40

TCTCAGACTT AGTCCATCAC ACTCTGTGCA TATGCCTGCT TGATGTGA

48

-112-

## (2) INFORMATION FOR SEQ ID NO:30:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CTCTCATCTG CTAGCACGCT CGAATAGTGT CAGTCGATGT GA

42

## 15 (2) INFORMATION FOR SEQ ID NO:31:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TACAGCGATT CACCCTTGTT TAAGGGTTAC ACCCATGTTA

40

## (2) INFORMATION FOR SEQ ID NO:32:

30

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

## (ii) MOLECULE TYPE: DNA (genomic)

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

40

-113-

ATCAGCGATT AACGCTTGT TCAATGTTAC ACCCATGTTA

40

(2) INFORMATION FOR SEQ ID NO:33:

5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

15 TTCAGCGATT AACGCTTATT TTAGCGTTAC ACCCATGTTA

40

(2) INFORMATION FOR SEQ ID NO:34:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATCAGCGATT CACCCCTTGTT TTAAGGTTGC ACCCATGTTA

40

30

(2) INFORMATION FOR SEQ ID NO:35:

35 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

40

-114-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ATCAGCGATT CACCCCTTGTT TAAAGCGTTAC ACCCATGTTG

40

## 5 (2) INFORMATION FOR SEQ ID NO:36:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- 10 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ATCAGCGATT CACCCCTTGTT TTAAGGTTAC ACCCATGTTA

40

## (2) INFORMATION FOR SEQ ID NO:37:

20

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

25

- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

30

ATCAGCGATT AACGCTTATT TTAGCGTTAC ACCCATGTTA

40

## (2) INFORMATION FOR SEQ ID NO:38:

35

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40

-115-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

5 ATCAGCGATT AACGCTTGTT TTAGTGTTC ACCCATGTTA

40

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

ATCAGCGATT AACGCTTATT TTAGCATTAC ACCCATGTTA

40

20 (2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 10 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GCCATGCTTT

10

35 (2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 10 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

-116-

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CTCTATTTCT

10

(2) INFORMATION FOR SEQ ID NO:42:

10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

20

TATGTGACGC TA

12

(2) INFORMATION FOR SEQ ID NO:43:

25

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

35

TATAGTCGTA

10

(2) INFORMATION FOR SEQ ID NO:44:

40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs

-117-

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

ATAGCGTATT A

11

10 (2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 13 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

ATAGTTACGT CAT

13

25 (2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 14 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA (genomic)

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

AATAGTGAAG TGTT

14

(2) INFORMATION FOR SEQ ID NO:47:

40

-118-

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

10

ATAGGCCCGG T

11

## (2) INFORMATION FOR SEQ ID NO:48:

15

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

25

AATAGTGAGG CTTG

14

## (2) INFORMATION FOR SEQ ID NO:49:

30

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: RNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GUAACUAGAG AU

12

## (2) INFORMATION FOR SEQ ID NO:50:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 98 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear
- 10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO  
              (ix) FEATURE:  
15            (A) NAME/KEY: misc\_feature  
              (B) LOCATION: 7..18  
              (D) OTHER INFORMATION: /note= "Position 7-18 is RNA; the  
                          remainder of the sequence is DNA."
- 20           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GGAAAAGUAA CUAGAGAUGG AAGAGATGGC GACNNNNNN NNNNNNNNNN NNNNNNNNNN 60  
NNNNNNNNNN NNNNNNNNNN NNNCGGTAAG CTTGGCAC 98

## 25           (2) INFORMATION FOR SEQ ID NO:51:

- 30           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 99 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear
- 35           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO  
              (ix) FEATURE:  
              (A) NAME/KEY: misc\_feature  
              (B) LOCATION: 1..24  
              (D) OTHER INFORMATION: /note= "Positions 1-24 is RNA; the  
                          remainder of the sequence is DNA."
- 40

-120-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GGAAAAAGUA ACUAGAGAUG GAAGAGATGG CGACNNNNNN NNNNNNNNNN NNNNNNNNNN 60  
NNNNNNNNNN NNNNNNNNNN NNNNCGGTAA GCTTGGCAC 99

5

## (2) INFORMATION FOR SEQ ID NO:52:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

20 CCAATAGTGC TACTGTGTAT CTCATGCTG GAAACACGGG TTATCTCCCG 50

## (2) INFORMATION FOR SEQ ID NO:53:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- 30 (ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

35 CCAAAACAGT GGAGCATTAT ATCTACTCCA CAAAGACCAC TTTCTCCCG 50

## (2) INFORMATION FOR SEQ ID NO:54:

40 (i) SEQUENCE CHARACTERISTICS:

-121-

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

ATCCGTACTA GCATGCAGAC AGTCTGTCTG CTTTTTCATT ACTCACTCCC

50

(2) INFORMATION FOR SEQ ID NO:55:

15

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 49 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

20

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

25

CAATTCACTGA TGACCAACTC TGTCAACACG CGAACTTTA ACACTGGCA

49

(2) INFORMATION FOR SEQ ID NO:56:

30

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 50 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

35

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

-122-

CTTCCACCTT CCGAGCCGGA CGAAGTTACT TTTTATCACA CTACGTATTG

50

## (2) INFORMATION FOR SEQ ID NO:57:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

## 15           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

GGCAAGAGAT GGCATATATT CAGGTAAC TGAGATACC CTGTCTGCCA

50

## (2) INFORMATION FOR SEQ ID NO:58:

- 20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear
- 25           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

- 30           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:
- CTAGACCATT CACGTTTACCA AAGCTATGGT AAGAACTAGA ATCACCGCGTA

50

## 35           (2) INFORMATION FOR SEQ ID NO:59:

- 40           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single

-123-

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

5 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

CGTACACGTG GAAAAGCTAT AAGTCAGTT CTCATCATGT ACCTGACCGC

50

10

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

25

CAGTGATAACA TGAGTGCACC GCTACGACTA AGTCTGTAAC TTATTCTACC

50

(2) INFORMATION FOR SEQ ID NO:61:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

-124-

ACCGAATTAA ACTACCGAAT AGTGTGGTTT CTATGCTTCT TCTTCCTGA

50

## (2) INFORMATION FOR SEQ ID NO:62:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

15           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

CAGGTAGATA TAATGCGTCA CCGTGCTTAC ACTCGTTTA TTAGTATGTC

50

## (2) INFORMATION FOR SEQ ID NO:63:

- 20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 49 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

25           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

30           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

CCCTACAACA CCACTGGGCC CAATTAGATT AACGCTATTT TATAACTCG

49

## 35           (2) INFORMATION FOR SEQ ID NO:64:

- (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 49 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

5 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CCAAACGGTT ATAAGACTGA AAACTCAATC AATAGCCAA TCCTCGCCC

49

10

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

25

CACATGTATA CCTAAGAAAT TGGTCCCGTA GACGTCACAG ACTTACGCCA

50

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

30

(A) LENGTH: 50 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(iii) MOLECULE TYPE: DNA (genomic)

(iv) HYPOTHETICAL: NO

(v) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

40

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CACAAACGAAA ACAATCTTCC TTGGCATACT GGGGAGAAAG TCTGTTGTCC

50

## (2) INFORMATION FOR SEQ ID NO:67:

## 5 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CACACGAACA TGTCCATTAA ATGGCATTCC GTTTTCGTT CTACATATGC

50

## 20 (2) INFORMATION FOR SEQ ID NO:68:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

30

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

CAGAACGAGG GTCTTGTAAG ACTACACCTC CTCAGTGACA ATAATCCTG

49

35

(2) INFORMATION FOR SEQ ID NO:69:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid

40

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (iii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

10 CACTACAGCC TGATATATAT GAAGAACAGG CAACAAGCTT ATGCACTGG

49

(2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (iii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

25 GGGTACATTT ATGATTCTCT TATAAAAGAGA ATATCGTACT CTTTTCCCCA

50

(2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30 (iii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

-128-

CCAAAGTACA TTCCAACCCC TTATACGTGA AACTTCCAGT AGTTTCCTA

49

## (2) INFORMATION FOR SEQ ID NO:72:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

## 15           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CTTGAAGATC CTCATAAGAC GATTAAACAA TCCACTGGAT ATAATCCGGA

50

## (2) INFORMATION FOR SEQ ID NO:73:

- 20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 25           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

## 30           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

CGAATAGTGT CCATGATTAC ACCAATAACT GCCTGCCTAT CATGTTTATG

50

## 35           (2) INFORMATION FOR SEQ ID NO:74:

- 40           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

5

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

10 CCAAGAGAGT ATCGGATACA CTTGGAACAT AGCTAACTCG AACTGTACCA

50

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 48 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

25

CCACTGATAA ATAGGTAACT GTCTCATATC TGCCAATCAT ATGCCGTA

48

(2) INFORMATION FOR SEQ ID NO:76:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

-130-

CCCAAATTAT AAACAATTAA ACACAAGCAA AAGGAGGTTTC ATTGCTCCGC

50

## (2) INFORMATION FOR SEQ ID NO:77:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

## 15           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

CAATAAACTG GTGCTAAACC TAATACCTTG TATCCAAGTT ATCCTCCCC

50

## (2) INFORMATION FOR SEQ ID NO:78:

- 20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear
- 25           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

30           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

CCGAATGACA TCCGTAGTGG AACCTTGCTT TTGACACTAA GAAGCTACAC

50

## 35           (2) INFORMATION FOR SEQ ID NO:79:

- 40           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single

-131-

## (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

5 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

CCRTAACAAA TACCATAGTA AAGATCTGCA TTATATTATA TCGGTCCACC

50

10

(2) INFORMATION FOR SEQ ID NO:80:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

25

CAGAACAAAG ATCAGTAGCT AAACATATGG TACAAACATA CCATCTGCA

50

(2) INFORMATION FOR SEQ ID NO:81:

## (i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 49 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

-132-

CCTTTAGTTA GGCTAGCTAC AACGATTTT CCCTGCTTGG CAACGACAC

49

## (2) INFORMATION FOR SEQ ID NO:82:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

15           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

CTCCCTACGT TACACCAGCG GTACGAATT TCCACGAGAG GTAATCCGCA

50

## (2) INFORMATION FOR SEQ ID NO:83:

- 20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 36 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 25           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

- 30           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CGGCACCTCT AGTTAGACAC TCCGGAATT TTCCCC

36

35           (2) INFORMATION FOR SEQ ID NO:84:

- 40           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 49 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single

-133-

## (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
5 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

CGGCACCTCT AGTTAGACAC TCCGGAATT TAGCCTACCA TAGTCCGGT

49

10

(2) INFORMATION FOR SEQ ID NO:85:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 47 base pairs  
15 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)  
20 (iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

25

CCCTTTGGTT AGGCTAGCTA CAACGATTTT TCCCTGCTTG AATTGTA

47

(2) INFORMATION FOR SEQ ID NO:86:

- (i) SEQUENCE CHARACTERISTICS:  
30 (A) LENGTH: 51 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35

- (ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

40

-134-

CCCTTTGGTT AGGCTAGCTA CAACGATTTT TCCCTGCTTG ACCTGTTACG A

51

## (2) INFORMATION FOR SEQ ID NO:87:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 48 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

15           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

CCTTTAGTTA GGCTAGCTAC AACGATTTTT CCCTGCTTGG AACGACAC

48

## (2) INFORMATION FOR SEQ ID NO:88:

- 20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

25           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO

30           (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

CATGGCTTAA TCATCCTCAA TAGAAGACTA CAAGTCGAAT ATGTCCCCCC

50

- 35           (2) INFORMATION FOR SEQ ID NO:89:

- 40           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid

-135-

- (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

5            (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

10 CAACAGAGCG AGTATCACCC CCTGTCAATA GTCTGTATGAA ACATTGGGCC

50

(2) INFORMATION FOR SEQ ID NO:90:

15 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 49 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

20           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

25 TACCGACBAG GGGAAATTAAA AGCTAGCTGG TTATGCAACC CTTTTCGCA

49

(2) INFORMATION FOR SEQ ID NO:91:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

-136-

CTCGAAACAG TGATATTCTG AACAAACGGG TACTACGTGT TCAGCCCC

49

## (2) INFORMATION FOR SEQ ID NO:92:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

## 15           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

CCAATAACGT AACCCGGTTA GATAAGCACT TAGCTAAGAT GTTTATCCTG

50

## (2) INFORMATION FOR SEQ ID NO:93:

- 20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 25           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

## 30           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

CAATACAATC GGTACGAATC CAGAAACATA ACGTTGTTTC AGAATGGTCC

50

## 35           (2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single

-137-

## (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

5 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

GCAACAAACAA GAACCAAGTT ACATACACGT TCATCTATAC TGAACCCCCA

50

10

(2) INFORMATION FOR SEQ ID NO:95:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 50 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

20

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

25

CCTTTGAGTT CCTAAATGCC GCACGGTAAG CTTGGCACAC TTTGACTGTA

50

(2) INFORMATION FOR SEQ ID NO:96:

30

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

-138-

CAAAGATCTC ACTTTGGAAA TGCATAATAT GTATATTCCG CCTGTCTGC

49

## (2) INFORMATION FOR SEQ ID NO:97:

- 5           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 10           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

15           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

CCACGTAGAA TTATCTGATT TATAACATAA CGCAGGATAA CTCTCGCCCA

50

## (2) INFORMATION FOR SEQ ID NO:98:

- 20           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 48 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single  
              (D) TOPOLOGY: linear

- 25           (ii) MOLECULE TYPE: DNA (genomic)  
              (iii) HYPOTHETICAL: NO  
              (iv) ANTI-SENSE: NO

30           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CACAAAGAAAG TGTCGTCTCC AGATATTGAA GTACPAGGAA CTACGCC

48

## 35           (2) INFORMATION FOR SEQ ID NO:99:

- 35           (i) SEQUENCE CHARACTERISTICS:  
              (A) LENGTH: 50 base pairs  
              (B) TYPE: nucleic acid  
              (C) STRANDEDNESS: single

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## (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
5 (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

CATGAAGAAA TAGGACATTC TACAGGCTGG ACCGTTACTA TGCCTGTAGG

50

10

## (2) INFORMATION FOR SEQ ID NO:100:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 46 base pairs  
15 (B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

20

- (iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

25

CATAGGATAA TCATGGCGAT GCTTATGACG TGTACATCTA TACCTT

46

## (2) INFORMATION FOR SEQ ID NO:101:

- (i) SEQUENCE CHARACTERISTICS:  
30 (A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35

- (ii) MOLECULE TYPE: DNA (genomic)  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

40

WO 98/49346

PCT/US98/08677

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CAGATGATCT TCCTTAAAG ACTACCCCTT AAAGAAACAT AAGGTACCCC

50

## What Is Claimed Is:

1. A catalytic DNA molecule having site-specific endonuclease activity specific for a nucleotide sequence defining a cleavage site in a preselected substrate nucleic acid sequence,  
5 said molecule having first and second substrate binding regions flanking a core region,  
wherein said first substrate binding region has a sequence complementary to a first portion of said  
10 preselected substrate nucleic acid sequence,  
said second substrate binding region has a sequence complementary to a second portion of said preselected substrate nucleic acid sequence, and  
said core region having a sequence according to the  
15 formula:

(I.) T(stem)'AGC(stem)"Z,

wherein said (stem)' and (stem)" are each three sequential nucleotides which when hybridized as a  
20 (stem)':(stem)" pair comprise three base pairs including at least two G:C pairs and wherein said Z = WCGR or WCGAA, and W = A or T and R = A or G; or

(II.) RGGCTAGGXACAAACGA (SEQ ID NO 122),

25

wherein said X = T, C or A, and R = A or G.

2. The molecule of claim 1 wherein said formula I defines SEQ ID NO 120 (8-17).
- 30 3. The molecule of claim 1 wherein said formula II defines SEQ ID NO 121 (10-23).
4. The molecule of claim 1 wherein said first or second substrate binding region is from 5 to 13 nucleotides

in length.

5. The catalytic DNA molecule of claim 1 wherein said catalytic DNA molecule comprises deoxyribonucleotides (DNA), modified DNA, nucleotide analogs, or composites thereof.

5 6. The catalytic DNA molecule of claim 1 wherein said substrate nucleic acid comprises RNA, DNA, modified RNA, modified DNA, nucleotide analogs, or composites thereof.

10 7. The catalytic DNA molecule of claim 1 wherein said catalytic DNA molecule comprises a single-stranded deoxyribonucleic acid having 5' and 3' termini, wherein said termini are modified with exonuclease-resistant nucleotides.

8. The catalytic DNA molecule of claim 7 wherein said exonuclease-resistant nucleotides comprise nucleoside phosphorothioate.

15 9. The catalytic DNA molecule of claim 1 wherein said first or second substrate binding region comprises at least two phosphorothioate nucleosides.

20 10. The catalytic DNA molecule of claim 1 wherein said core region comprises a phosphorothioate nucleoside residue on a dipyrimidine within said core.

11. The catalytic DNA molecule of claim 7 wherein said 3' termini comprises an inverted (3',3'-linked) nucleotide.

25 12. The catalytic DNA molecule of claim 1 wherein said catalytic DNA molecule comprises a 2' O-methyl ribonucleotide.

13. The catalytic DNA molecule of claim 1 wherein said first and second substrate binding regions comprise a nucleotide sequence complementary to a sequence selected from the group consisting of SEQ ID NOS 102-119.

30 14. The catalytic DNA molecule of claim 1 wherein said molecule catalyzes a reaction with a  $K_m$  of about 0.05 - 1000 nanomolar.

15. The catalytic DNA molecule of claim 1 wherein said

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catalytic DNA molecule binds said substrate with a  $K_m$  of less than about 1.0 micromolar.

5        16. The catalytic DNA molecule of claim 1 wherein said catalytic DNA molecule binds said substrate with a  $K_m$  of about 0.1 nanomolar.

10      17. The catalytic DNA molecule of claim 1 wherein said molecule has a catalytic reaction turnover rate ( $k_{cat}$ ) of about 0.005 - 0.1 min<sup>-1</sup>.

15      18. The catalytic DNA molecule of claim 1 wherein said endonuclease activity is enhanced by the presence of a divalent cation.

19. The catalytic DNA molecule of claim 18 wherein said divalent cation is selected from the group consisting of Pb<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, and Ca<sup>2+</sup>.

20      20. The catalytic DNA molecule of claim 18 wherein said endonuclease activity is enhanced by the presence of Mg<sup>2+</sup>.

21. The catalytic DNA molecule of claim 1 wherein said endonuclease activity is enhanced by the presence of a monovalent cation.

22. The catalytic DNA molecule of claim 21, wherein said monovalent cation is selected from the group consisting of Na<sup>+</sup> and K<sup>+</sup>.

25      23. A composition comprising two or more populations of catalytic DNA molecules according to claim 1, wherein each population of catalytic DNA molecules is capable of cleaving a different nucleotide sequence in a substrate.

30      24. A composition comprising two or more populations of catalytic DNA molecules according to claim 1, wherein each population of catalytic DNA molecules is capable of recognizing a different substrate.

25. A method of cleaving a target nucleic acid molecule, comprising:

5 a) admixing a catalytic DNA molecule according to claim 1 with a target nucleic acid molecule having a preselected substrate nucleic acid sequence to said first and second substrate binding regions, to form a reaction admixture; and

10 b) maintaining said admixture under predetermined reaction conditions to allow said catalytic DNA molecule to cleave said target nucleic acid molecule, thereby producing a population of substrate products.

26. The method of claim 25, wherein said substrate comprises RNA.

15 27. The method of claim 25, wherein said predetermined reaction conditions include the presence of a monovalent cation, a divalent cation, or both.

28. The method of claim 25 wherein said admixing comprises introducing said catalytic DNA molecule into a cell containing said target nucleic acid molecule.

20 29. A method of engineering a catalytic DNA molecule that cleaves a preselected substrate nucleic acid sequence in a target nucleic acid molecule, comprising the steps of:

25 a) selecting a substrate nucleic acid sequence of from 10 to 26 nucleotides in length in a target nucleic acid molecule; and

b) synthesizing a deoxyribonucleic acid molecule comprising first and second substrate binding regions flanking a core region,

30 wherein said first substrate binding region has a sequence complementary to a first portion of said preselected nucleic acid target sequence,

said second substrate binding region has a sequence

complementary to a second portion of said preselected nucleic acid target sequence, and  
said core region having a sequence according to the formula:

5 (I.) T(stem)'AGC(stem)"Z,

wherein said (stem)' and (stem)" are each three sequential nucleotides which when hybridized as a (stem) ':(stem)" pair comprise three base pairs including at 10 least two G:C pairs and wherein said Z = WCGR or WCGAA, and W = A or T and R = A or G; or

(II.) RGGCTAGCXACAACGA (SEQ ID NO 122),

15 wherein said X = T, C or A, and R = A or G.

30. The method of claim 29 wherein said formula I defines SEQ ID NO 120 (8-17).

31. The method of claim 29 wherein said formula II 20 defines SEQ ID NO 121 (10-23).

32. The method of claim 29 wherein said first or second substrate binding region is from 5 to 13 nucleotides in length.

33. The method of claim 29 wherein said catalytic DNA 25 molecule comprises deoxyribonucleotides (DNA), modified DNA, nucleotide analogs, or composites thereof.

34. The method of claim 29 wherein said catalytic DNA molecule comprises a single-stranded deoxyribonucleic acid having 5' and 3' termini, wherein said termini are modified 30 with exonuclease-resistant nucleotides.

35. The method of claim 7 wherein said exonuclease-resistant nucleotides comprise nucleoside phosphorothioate.

36. The method of claim 29 wherein said

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first or second substrate binding region comprises at least two phosphorothioate nucleosides.

37. The method of claim 29 wherein said core region comprises a phosphorothioate nucleoside residue 5 on a dipyrimidine within said core.

38. The method of claim 34 wherein said 3' termini comprises an inverted (3',3'-linked) nucleotide.

39. The method of claim 29 wherein said catalytic DNA molecule comprises a 2' O-methyl ribonucleotide.

10 40. The method of claim 29 wherein said first and second substrate binding regions comprise a nucleotide sequence complementary to a sequence selected from the group consisting of SEQ ID NOS 102-119.

15 41. The method of claim 29 wherein said molecule catalyzes a reaction with a  $K_m$  of about 0.05 - 1000 nanomolar.

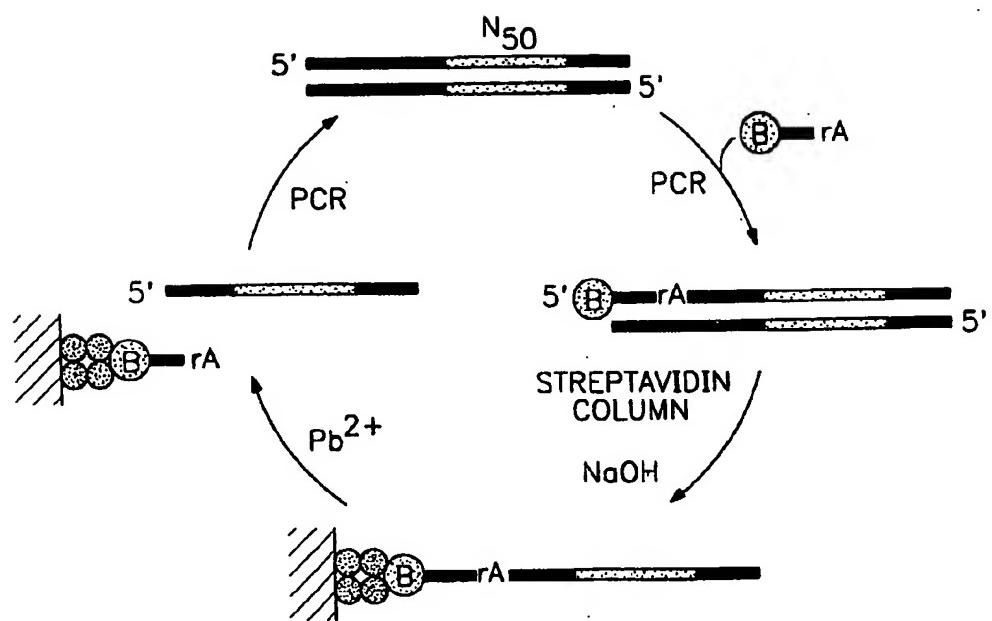
42. The method of claim 29 wherein said molecule has a catalytic reaction turnover rate ( $k_{cat}$ ) of about 0.005 - 0.1 min<sup>-1</sup>.

20 43. The method of claim 29 wherein said endonuclease activity is enhanced by the presence of a divalent cation.

44. The method of claim 43 wherein said divalent cation is selected from the group consisting of Pb<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, and Ca<sup>2+</sup>.

25 45. The method of claim 29 wherein said endonuclease activity is enhanced by the presence of a monovalent cation.

46. The method of claim 45, wherein said monovalent cation is selected from the group consisting of Na<sup>+</sup> and K<sup>+</sup>.

**FIG. 1**

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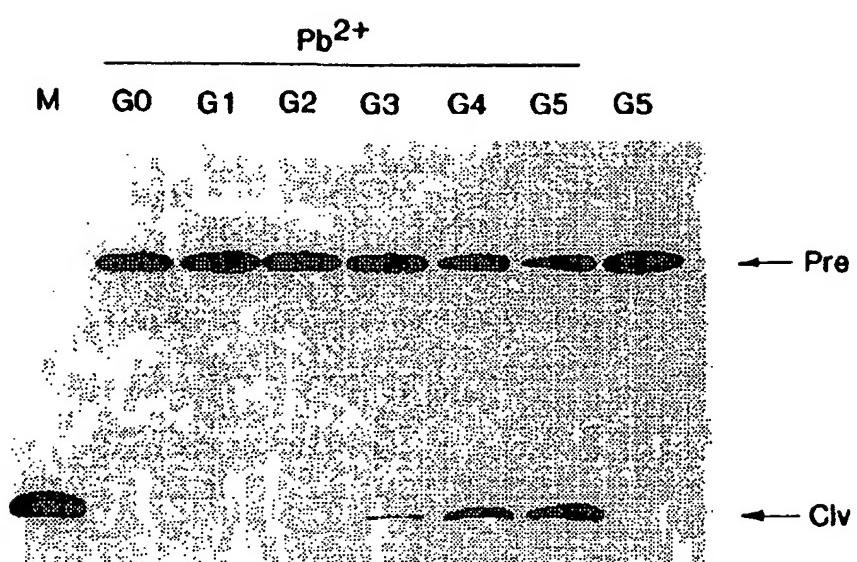


FIG. 2

TARGET RIBOADENYLATE			5'- CAGCCGATAGAGAAAAA - 5'			..... ATATCACTCAGATAATCTTAAGCAAGA - 5'		
SEQ ID NO.	# of clones	Substrate	5'- TCACAGATTCTCT	GAAGT	CGGC	TAT - GTGA - CGCTA	GGGGTCGCC	- 3'
14	8		5'- GGGGGAAACGCCGAAACAA [G - C]CT	GAACT	CGG	TAT - GTTA - ... CGTA	GGGGTCGCC	- 3'
15	3		5'- CGGGACTCCGTAGCCCA [T]GGCTTTT - .	GC	CGG	TAT - AGT - ... CGTA	GGGGTCGCC	- 3'
16	2	5'-	5'- CGGGACTCCGTAGCCCA [T]GGCTTTT - .	GC	CGG	- ATAG - ... CGTATTA	GGGGTCGCC	- 3'
17	2		5'- CCACCA[GTCCTTC]TC - .	G	AGCCG	AAC	- ATAGTTA - CGTCAT	ACETCCCGTAT - 3'
18	1		5'- ACCAGA[TTACTA-CT- -	ACC	AGCG	GTA	[A]ATAGTGAA - GTGTT	CGTACTAT - 3'
19	1		5'- ATAG[CCCATGGCTT	GGCT	AGCG	GCAC	[T]ATAGTG - ... TA	CCTGCCCTATCG - 3'
20	1		5'- TCTGCTCTCTCTATCT - .	ACCAAGTC	AGCC	AAATATGT	CGA	- ATAGT - ... CG - GT - 3'
21	1		5'- TTGCCCAGGATA[GTCGGCA - - - -	GACCTGGGTT	AGCG	ACA	- ATAG - G - CCCG - GT - 3'	
22	1		5'- TTCTTAGCTCGGCTA[A]CTCT	GT	AGCG	CAAC	AATAGTGAG - GC - TIG	A - 3'

CONSERVED REGIONS →  
BASE-PAIR-FORMING REGIONS (INDIVIDUALLY BOXED SEQUENCES)

BASE-PAIR-FORMING REGIONS (INDIVIDUALLY BOXED SEQUENCES)

3  
FIG.

**SUBSTITUTE SHEET (RULE 26)**

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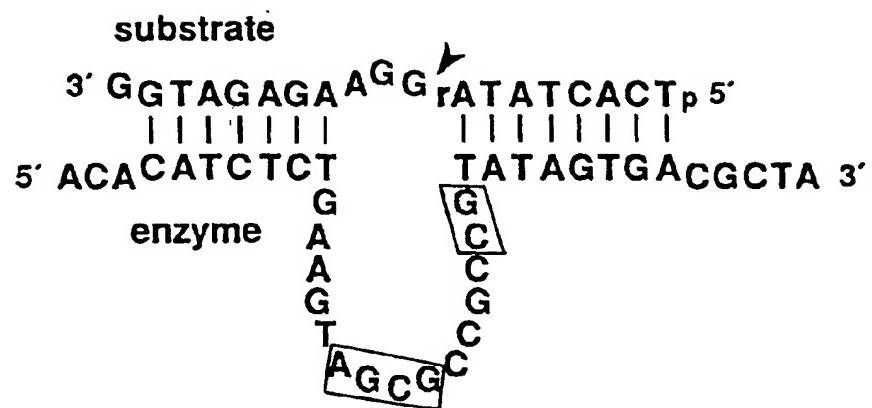


FIG. 4A

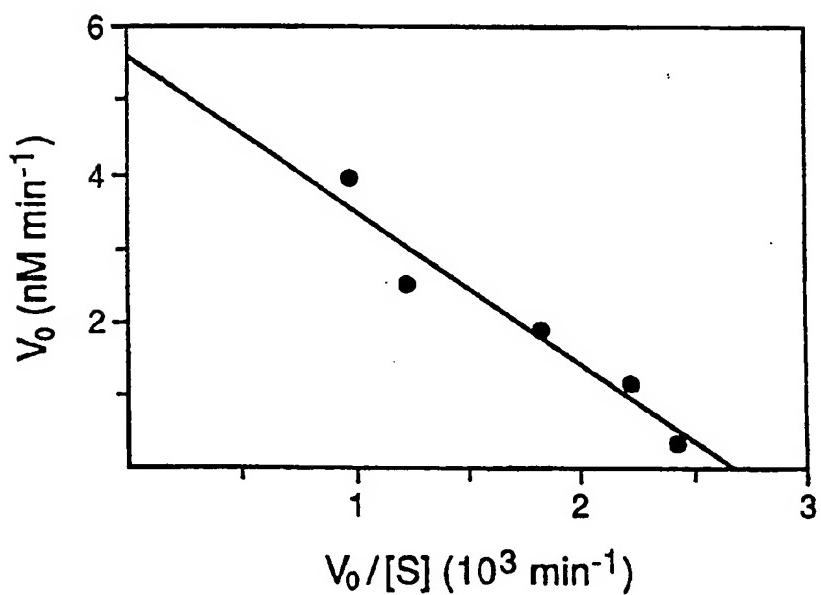


FIG. 4B

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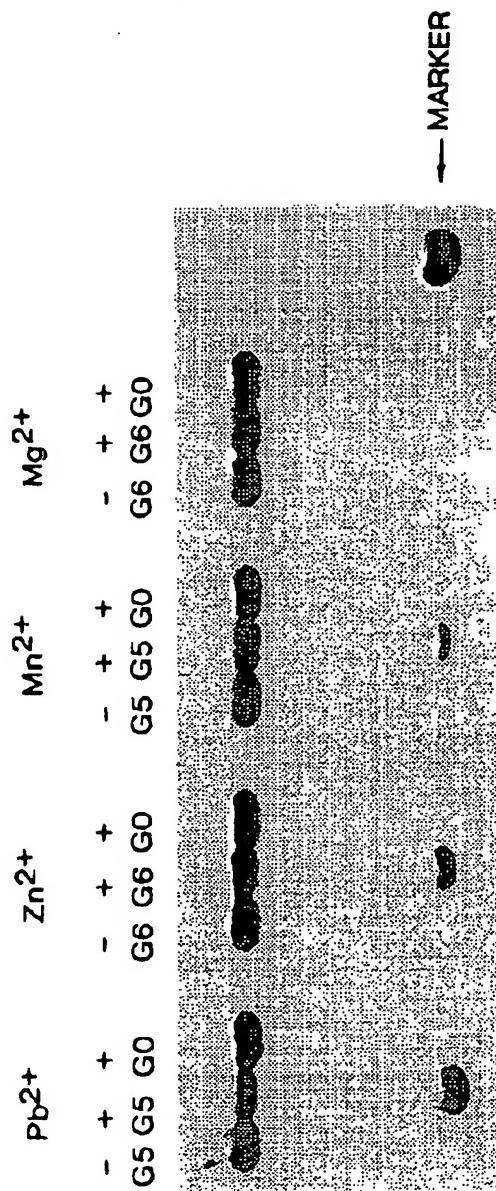


FIG. 5

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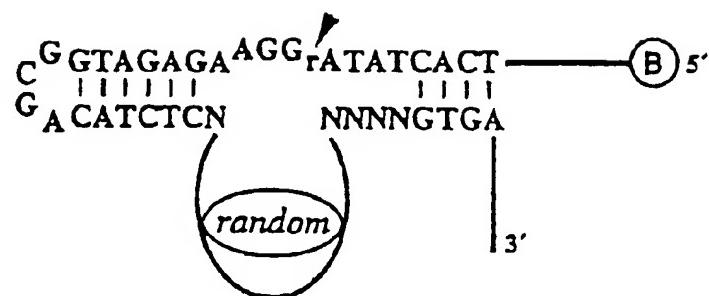


FIG. 6A

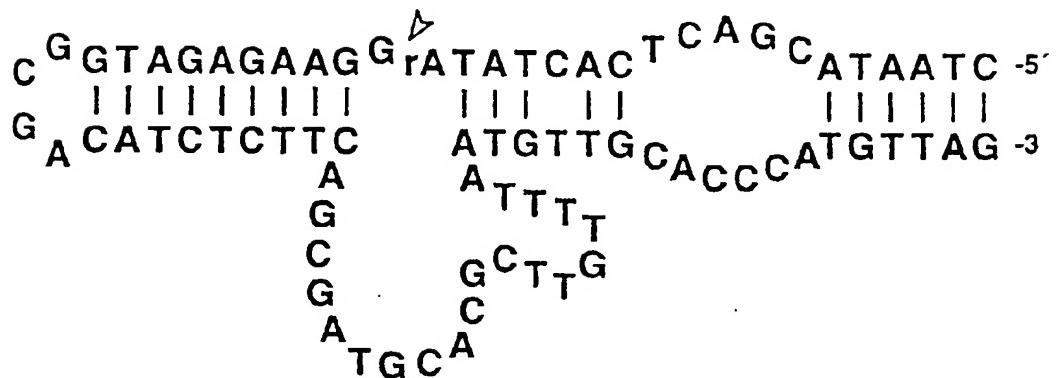
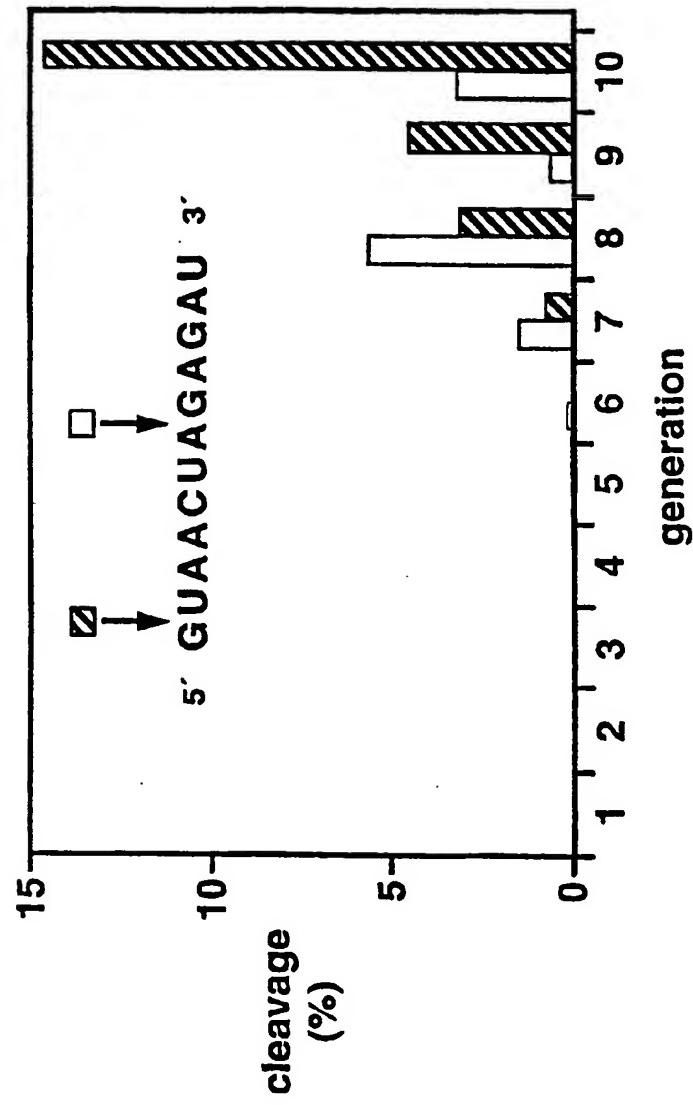


FIG. 6B

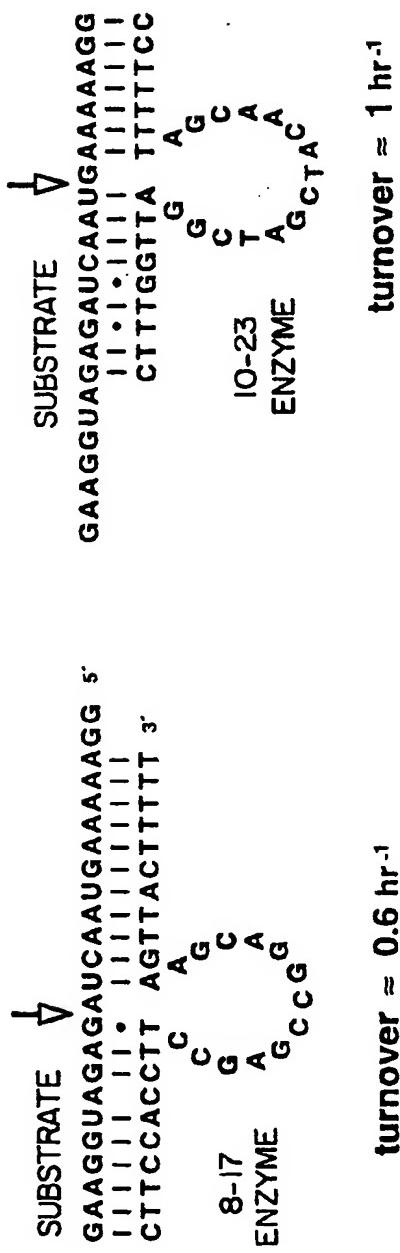
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10 mM Mg<sup>2+</sup>, pH 7.5, 37 °C, 2 h

FIG. 7

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turnover  $\approx$  1 hr<sup>-1</sup>

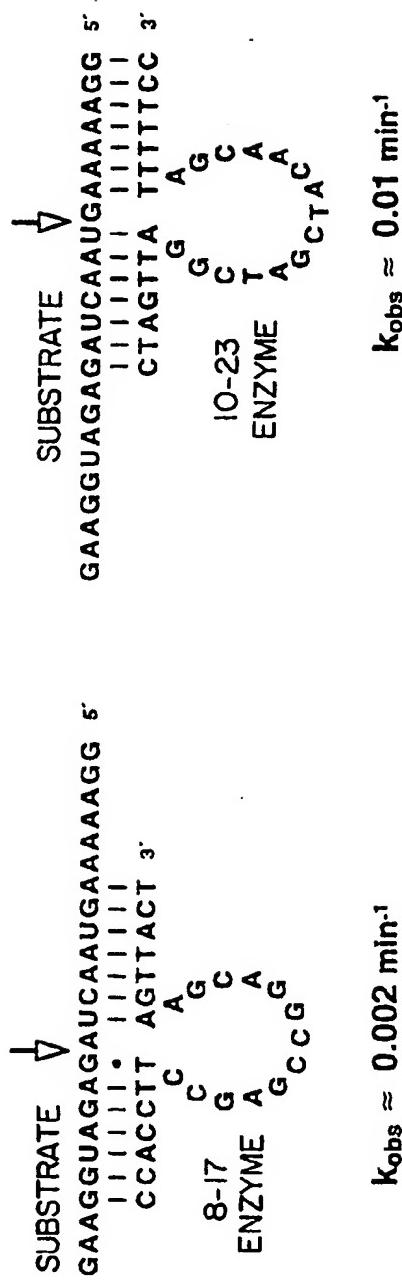
turnover = 0.6 hr<sup>-1</sup>

10 mM Mg<sup>2+</sup>, pH 7.5, 37 °C

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G  
正

**SUBSTITUTE SHEET (RULE 26)**

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$$k_{\text{obs}} = 0.01 \text{ min}^{-1}$$

$$k_{\text{obs}} \approx 0.0002 \text{ min}^{-1}$$

10 mM Mg<sup>2+</sup>, pH 7.5, 37 °C

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**SUBSTITUTE SHEET (RULE 26)**

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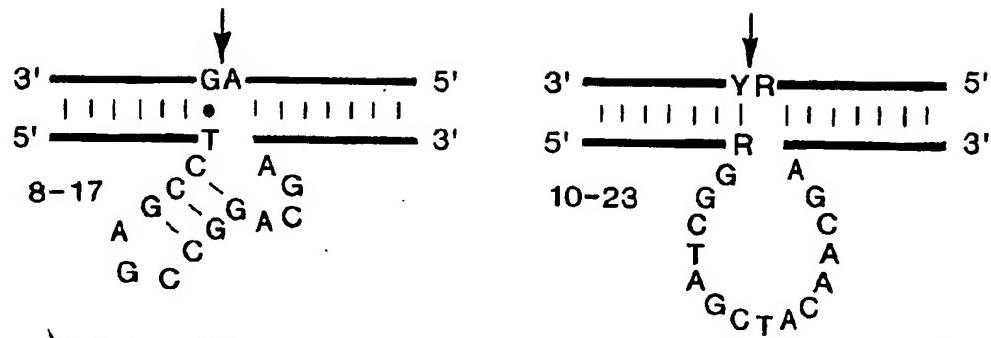


FIG. 10

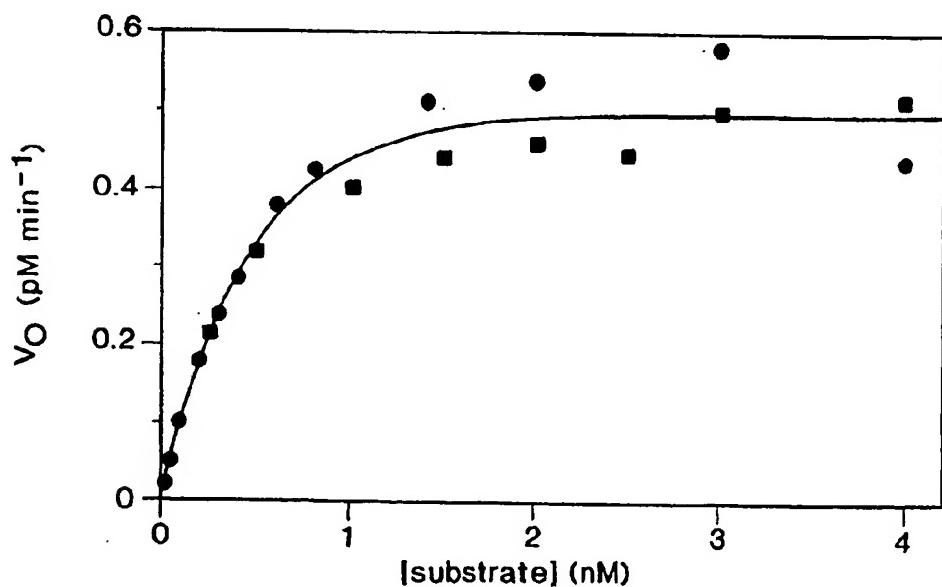


FIG. 11

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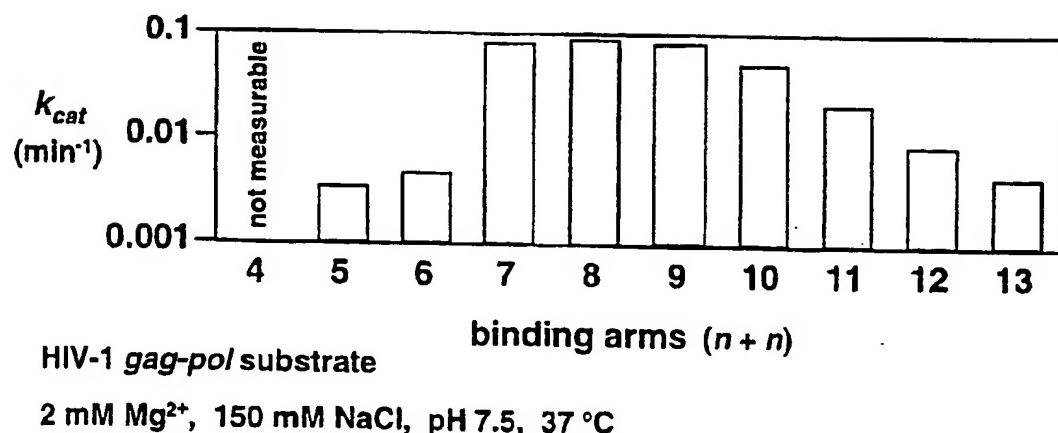


FIG. 12A

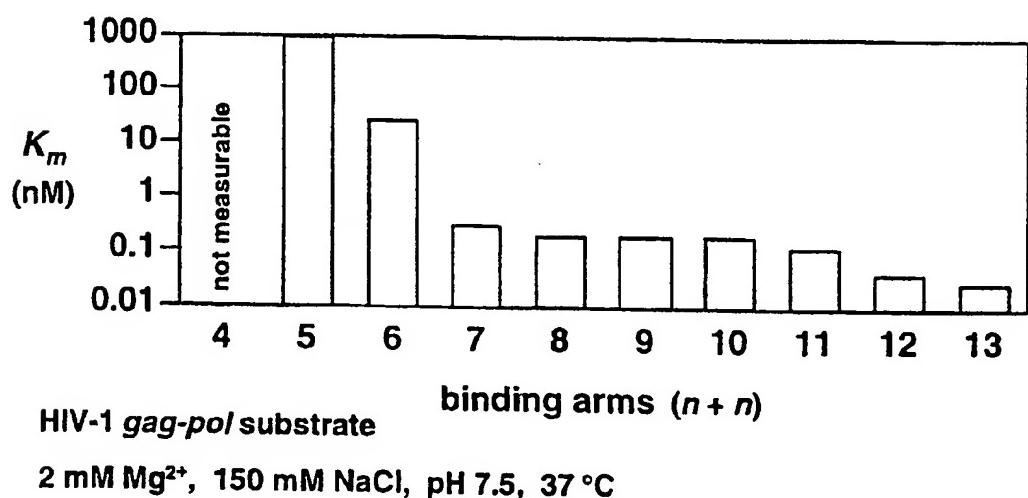


FIG. 12B

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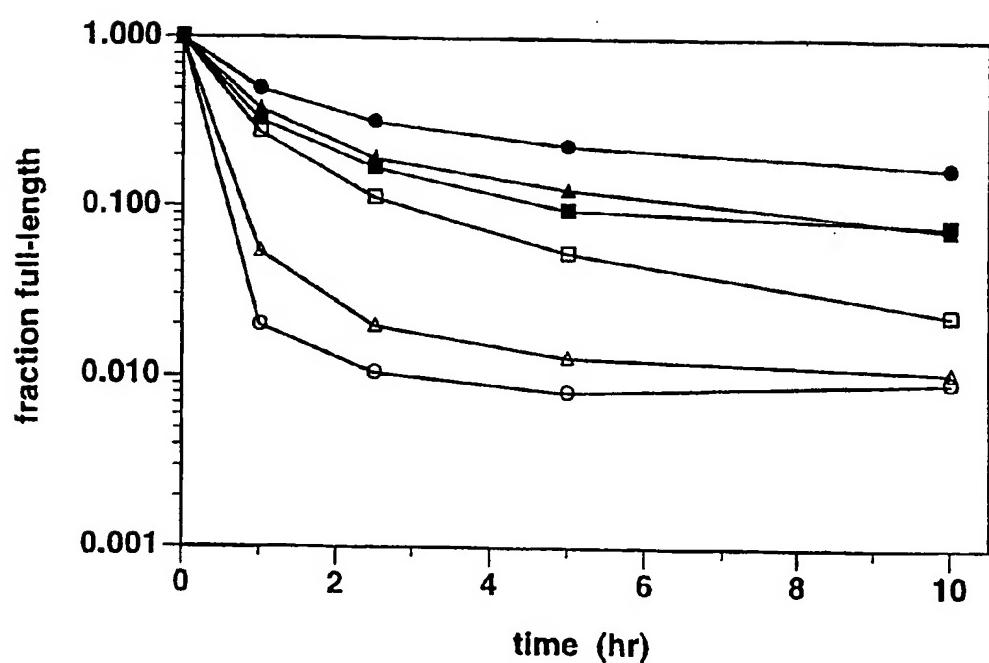


FIG. 13

## INTERNATIONAL SEARCH REPORT

International application No. PCT/US98/08677
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**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12Q 1/68; C12N 9/22; C07H 21/04  
US CL :435/6, 91.31, 172.1; 536/23.1, 23.2, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.31, 172.1; 536/23.1, 23.2, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN: MEDLINE, BIOSIS, EMBASE, SCISEARCH, WPIDS, CAPLUS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	BREAKER, R.R. DNA enzymes. Nature Biotechnology. May 1997, Vol. 15, pages 427-431, see entire document.	1-46
A	BURGSTALLER, P. et al. Synthetic Ribozymes and the First Deoxyribozyme. Angew. Chem. Int. Ed. Engl. 1995, Vol. 34, No. 11, pages 1189-1192, see entire document.	1-46

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
16 JUNE 1998	22 JUL 1998

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Andrew Wang</i> ANDREW WANG Telephone No. (703) 308-0196
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